Prognostic Biomarkers of Periodontal Disease

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PROGNOSTIC BIOMARKERS OF PERIODONTAL DISEASE

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Thesis Submitted to King's College London for the Degree of Doctor of Philosophy (PhD)

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London

2015

ABSTRACT

Objectives: Previous studies in our laboratory have identified the antimicrobial proteins Human Neutrophil Protein 1 – 3 (HNP1-3), Myeloid Related Protein 8 (S100A8/MRP8) and LL-37 as putative periodontal salivary biomarkers. The aims of the studies reported in this thesis were to investigate the diagnostic and prognostic potential of these markers, together with Matrix Metalloproteinase- 8 (MMP-8), serum markers C - reactive protein (CRP) and Interleukin-6 (IL-6), on the initial outcome of nonsurgical periodontal treatment.

Material & Methods: We carried out a cross sectional study which aimed to verify and establish a diagnostic threshold for a group of salivary biomarkers (MMP-8, HNP1-3, S100A8 and LL-37) and to test the validity of the diagnostic utility of these biomarkers. A total of 133 unstimulated saliva samples (46 with chronic periodontitis, 38 with aggressive periodontitis and 49 with gingivitis) were analysed by ELISA. In addition multiple markers were combined to give a single combined cut off point by normalising each biomarker to percentage of cut-off point value, (such that x + y = combined cut off point). These pre-determined cut-off points were applied to salivary AMPs levels in an independent cohort originally collected to investigate the effects of diabetes on periodontitis.

To investigate prognostic potential a total of 66 participants were recruited to a longitudinal intervention study of patients with moderate–severe Chronic Periodontitis. 53 subjects completed the protocol and were included in the final analysis. Subjects (28 male, 25 female) age range (23-65 years) with were recruited, with 14 smokers and 3 with type II diabetes, and saliva and serum samples were collected prior to periodontal examination. Patients were then given a course of non-surgical periodontal therapy over 2 visits. 8-10 weeks post-operatively saliva/serum sampling and clinical examination were repeated. Salivary MMP-8, S100A8 and HNP1-3 concentrations were all determined by ELISAs. In addition we measured serum levels of CRP and Interleukin 6.

Results: In the cross-sectional study the HNP1-3 and S100A8 could differentiate between gingivitis and chronic periodontitis with high specificity (around 90%) and around 75% sensitivity compared to MMP-8 which was able to discriminate between gingivitis and periodontitis (chronic and aggressive) with both high

specificity and sensitivity. LL37 showed no significant diagnostic potential. Within the independent cohort the application of pre-determined thresholds, either individual or combined cut-offs, were able to detect periodontitis with specificity of between 75 – 85 % but with very low sensitivity. In addition diabetic status was found to result in significantly increased MMP-8 and S100A8 concentrations in subjects with periodontal disease.

In the intervention study, treatment resulted in reductions in the mean: a) number of deep sites (>4mm) (33.57 ± 20.75 vs 18.51 ± 13.87; mean ± SD, p<0.0001); b) probing pocket depths (5.92 ± 0.47 mm vs 4.74 ± 0.76 mm, p<0.0001); c) bleeding index (0.32 ± 0.20 vs 0.21 ± 0.16, p<0.0001); d) plaque index (0.46 ± 0.20 vs 0.37 ± 0.18, p=0.0003). Only the mean concentrations of MMP-8 and S100A8 showed significant reductions post-treatment (MMP-8: 355.4 ± 319.9 ng/ml vs 216.6 ± 217.2 ng/ml, p<0.0001), (S100A8: 1182 ± 1095 ng/ml vs 693.9 ± 719.6 ng/ml, p=0.0007). Only the change in concentrations of MMP-8 were strongly associated with magnitude of treatment response (MMP-8: r^2= 0.1, p=0.02). In addition, the baseline levels of MMP-8 & S100A8 were also associated with treatment response (MMP-8: r^2= 0.1, p=0.03; S100A8: r^2=0.1, p=0.02). Overall, there were 13 out of 53 participants who did not respond to the treatment (24.5% of cases). MMP-8 baseline concentrations were significantly higher in responders (419.3 ± 343.1 ng/ml) than non-responders (158.8 ± 73.3 ng/ml) (p=0.009). MMP-8 concentrations at baseline that were above the cut-off (<182.8 ng/ml) predicted a good response to periodontal treatment with 77% sensitivity and 70% specificity. There was no effect of the single round of non-surgical periodontal treatment on the levels of systemic markers CRP & IL-6, and also there was no correlation between local and systemic markers.

Conclusion: These results of both studies suggest that MMP-8, HNP1-3 and S100A8 may be useful to identify cases of periodontitis with good specificity and moderate sensitivity and may give superior results when combined. In addition the salivary MMP-8 and S100A8 showed promising periodontal prognostic ability to detect the likelihood of a good response to treatment, with MMP-8 showed the best results with moderate sensitivity and specificity. However, further validation studies would be useful in larger, non-diabetic cohorts.

AKNOWLEDGEMENTS

My sincere gratitude goes to Allah who gave me the strength and health to accomplish my dream academically, and for the Ministry of Higher Education in my country “Libya” for funding my study in the UK.

I would like to express my extreme appreciation and respect to my first supervisor, Prof Francis Hughes, for his enormous support and guidance along my PhD journey. I could never have a better supervisor who is caring, kind and patient with his students.

I would like to thank my second supervisor, Dr. Mark Ide, for his guidance.

I would also like to thank my colleagues Dr. Tonnie Mulli, Dr. Omar Patel, Dr. Najam Imam, Dr. Wan-Chien Cheng and Mrs Paula Holloway, the lab manager, for their support and help during my lab work.

Many thanks to Dr. Penny Hodge and her colleagues, from University of Glasgow, for generously donating their collection of saliva samples.

I would like to sincerely thank all my fellow PhD students for their friendship and advice along the way, all the patients who kindly participated in my studies, and all the clinical/IT staff and students at Guy`s Hospital/KCL Dental Institute for their cooperation during my clinical work.

Last but never least, a huge thank to my great family especially my beloved parents for their support and prays, to my supportive husband Abdouldaim who never let me give up achieving my goal and to my angel Nezar for all the love and laughter he brought to our life. Not to forget my one and only sister Balsam for her continuous support.

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ABBREVIATIONS

%	Percentage
®	Registered trade mark
°C	Degree Celsius
A.a	Aggregatibacter actinomycetemcomitans
AAP	American Academy of Periodontology
ACVD	Atherosclerotic Cardiovascular Disease
AgP	Aggressive Periodontitis
AL	Attachment Level
AMP	Antimicrobial Peptide
ANOVA	Analysis Of Variance
APVT	Any Positive Value Test
AUC	Area Under the Curve
BI	Bleeding Index
BK	Belkais Karim
β-NAH	beta-N-acetyl Hexosaminidase
BoP	Bleeding on Probing
CAL	Clinical Attachment Loss
CDC	Centre for Disease Control and prevention
CP	Chronic Periodontitis
CRP	Circulating Reactive Protein
CVD	Cardiovascular Disease
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EFP	European Federation of Periodontology
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
FPR	False Positive Rate
G	Gingivitis
GCF	Gingival Crevicular Fluid
HbA1c	Glycosylated haemoglobin
HGF	Hepatocyte Growth Factor
HNP1-3	Human Neutrophil Peptide 1,2 & 3
Hrs	Hours
ICAM	Intercellular Adhesion Molecule
ICTP	Type I Collagen carboxyterminal Telopeptide
Ig	Immunoglobulin

IL	Interleukin
Inc.	Incorporation
INF-α	Interferon α
LC-MS/MS	Liduid Chromatography quadruple Mass Spectrometry
LL-37	Cathelicidin LL-37
LOC	Lab On Chip
LTB_4	Leukotriene B4
Ltd	Limited
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MMP	Matrix Metalloproteinase
MRP-8	Myeloid-related Protein 8
NDM	Normalised Data Method
ng	nanogram
NHS	National Health Service
OHI	Oral Hygiene Instruction
P	Parotid
PAF	Platelet Activating Factor
PI	Plaque Index
P. intermedia	Prevotella intermedia
P.g	Porphyromonas gingivalis
PD	Periodontal Disease
PDL	Periodontal Ligament
PhD	Doctor of Philosophy
pg	Picogram
PGE_2	Prostaglandin E 2
PMN	Polymorphnuclear cells
POC	Point Of Care
PPD	Probing Pocket Depth
r / r^2	Correlation coefficient
RA	Rheumatoid Arthritis
R & D	Research and Development
RANTES	Regulated on Activation Normal T-cell Expressed And secreted
Rec	Recession
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
rpm	revolutions per minute

S100A8	Protein S100A8
SD	Standard Deviation
Sec	Second
SELDI	Surface Enhanced Laser Desorption Ionization
SL	Sublingual
SM	Submandibular
T. forsythus	Tannerella forsythus
TIMP	Tissue Inhibitor of Metalloproteinase
TGF	Transforming Growth Factor
TM	Trade Mark
TNF-α	Tumor Necrosis Factor α
TOF-MS	Time Of Flight Mass Spectrometry
TPR	True Positive Rates
UK	United Kingdom
USA	United States of America
VEGF	Vasoactive Endothelial Growth Factor
vs	versus
WS	Whole Saliva
µl	Microlitre

CHAPTER 1- LITERATURE REVIEW

1. Literature Review

1.1 Introduction

Periodontal disease is a chronic, site-specific, cyclic disease of multi-factorial aetiology. The disease is a result of imbalance between pro-inflammatory and anti-inflammatory signals in response to the accumulation of dental plaque on the tooth surface adjacent to the gingival margin. In common with many other chronic diseases, periodontitis is characterised by periods of active tissue destruction and quiescence.

Traditionally, both diagnosis and assessment of prognosis of periodontal disease are based on clinical measures including probing pocket depth (PPD), clinical attachment loss (CAL), bleeding on probing (BOP), and radiographic assessment. However, these measures are of limited use for disease classification, assessing current disease activity, or predicting disease progression. Therefore, there is increasing interest in using additional diagnostic/prognostic markers in recent years.

Generally, there are three ways that have been used to assess damage to the periodontal tissues clinically: detection of bone loss radiographically, measurement of clinical attachment loss, and the visual detection of any signs of tissue destruction (Armitage, 1996). Most of the criteria that are used to assign either diagnosis or prognosis of periodontal disease are tooth-related rather than subject-related. Therefore, it would be useful to look for markers at the patient level that could help in monitoring health status, disease onset, treatment response and outcome.

In addition, it can still be a big challenge for the clinician to identify and treat sites and patients that do not respond as expected to periodontal therapy provided. Therefore, this PhD project aims to investigate potential biomarkers that might predict these different outcomes to periodontal therapy on a patient level, through longitudinal study of the changes in biomarkers before and after non-surgical periodontal treatment, and the relationship of these measures to clinical outcomes of treatment.

1.1.1 Classification and Diagnosis of Periodontal Diseases

Plaque-induced periodontal diseases have traditionally been categorised as either gingivitis or periodontitis. Both plaque-induced gingivitis and periodontitis are by far the most frequent of all forms of diseases that may affect the periodontal tissues.

The current classification system of periodontal diseases was developed in 1999 at the International Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999), in which periodontal diseases are classified into eight main categories as shown in Table 1-1.

	Periodontal Disease Category	Descriptive Criteria
I	**Gingival Diseases**	a. Dental plaque-induced gingival diseases* b. Non-plaque-induced gingival lesions
II	**Chronic Periodontitis**	a. Localized(≤30% of sites involved) b. Generalized(>30% of sites involved) • Slight: 1-2mm CAL** • Moderate: 3-4mm CAL • Severe: ≥5mm CAL
III	**Aggressive Periodontitis**	a. Localized(≤30% of sites involved) b. Generalized(>30% of sites involved) • Slight: 1-2mm CAL • Moderate: 3-4mm CAL • Severe: ≥5mm CAL Usually patients below 35 years of age are affected
IV	**Periodontitis as a Manifestation of Systemic Disease**	a. Associated with haematological disorders b. Associated with genetic disorders c. Not otherwise specified
V	**Necrotizing Periodontal Diseases**	a. Necrotizing ulcerative gingivitis b. Necrotizing ulcerative periodontitis
VI	**Abscesses of the Periodontium**	a. Gingival abscesses b. Periodontal abscesses c. Pericoronal abscesses
VII	**Periodontitis Associated with Endodontic Lesions**	a. Combined periodontic-endodontic lesions
VIII	**Developmental or Acquired Deformities and Conditions**	a. Localized tooth-related factors that modify or predispose plaque-induced gingival diseases/periodontitis b. Mucogingival deformities and conditions around teeth c. Mucogingival deformities and conditions on edentulous ridges d. Occlusal trauma

* Can occur on a periodontium with no attachment loss or on a periodontium with attachment loss that is not progressing.

**CAL= Clinical Attachment Loss

Table 1-1:Abbreviated version of the 1999 Classification of Periodontal Diseases and Conditions (Armitage, 1999).

Gingivitis

Gingivitis is a reversible inflammation of the gingival tissue without loss of attachment or alveolar bone. It can also occur in a reduced but stable periodontium (Highfield, 2009). Usually it represents the body's reaction toward exposure of the gingival tissues to bacterial plaque (Mariotti, 1999). Classic studies of experimental gingivitis have demonstrated that clinical gingivitis develops within 21 days of ceasing oral hygiene practices and that gingival health is restored when these oral hygiene measures reinstituted (Lőe et al., 1967; Theilade et al., 1966). Interestingly, the natural history of gingivitis frequently does not result in progression to periodontitis (Lindhe et al., 1975).

Chronic periodontitis

(ChP) is a chronic inflammatory condition of the tooth-supporting tissues, which is characterised by apical migration of epithelial attachment along with alveolar bone destruction and loss of marginal periodontal ligament. It is cyclical in nature with long periods of quiescence interspersed with shorter periods of destruction (Socransky et al., 1984).

The main characteristics of chronic periodontitis, according to the 1999 classification of periodontal conditions (Armitage, 2004b) are :

- Prevalent in adults but may occur in children.
- The amount of tissue destruction is related to the presence of local factors
- Subgingival calculus present at diseased sites.
- Usually has slow to moderate rate of progression.
- Further tissue destruction may occur if the diseased sites are left without treatment

The severity of this periodontal disease may vary from one patient to another and from site to site Table 1-1.

Aggressive Periodontitis

Aggressive periodontitis (AgP) comprises a group of uncommon forms of periodontitis that tend to start at an early age and may aggregate in families.

The common features of AgP are (Lang et al., 1999):

- The patients are clinically healthy (Except for the presence of periodontitis).
- Rapid attachment loss and bone destruction.
- Familial aggregation.

There are secondary features which are not present in all AgP cases (Lang et al., 1999):

- The amount of dental plaque is inconsistent with the severity of periodontal tissue destruction.
- High proportion of *Aggregatibacter actinomycetemcomitans (A.a*), and in some Far East populations, *Porphyromonas gingivalis (P.g)*.
- Phagocyte abnormalities.
- Hyper-responsive macrophage phenotype.
- Attachment and alveolar bone loss progression may be self-arresting.

There are two forms of AgP; localised and generalised Table 1-2.

Criteria	Localised AgP	Generalised AgP
Onset	Circumpubertal	Usually affect individuals under 30 years of age, but can affect older
Serum antibody response to infecting agent	Robust	Poor
Distribution	1st molar/incisor with interproximal attachment loss on at least 2 permanent teeth (including a 1st molar) & not more than 2 teeth other than 1stmolars & incisors	Interproximal attachment loss affecting at least 3 permanent teeth other than 1st molars & incisors

Table 1-2:showing comparison between the two forms of Aggressive Periodontitis (Lang et al., 1999; Tonetti and Mombelli, 2003).

Differentiating AgP from ChP can be challenging clinically in some cases. However, the main differences are the rate of progression, the pattern of alveolar bone destruction, the amount of local factors including plaque and the age of onset or detection (Armitage, 1996; Armitage et al., 2010; Lang et al., 1999). Despite all the effort and time that has been invested to classify and diagnose periodontal disease, there is still no perfect classification that can sharply distinguish between different forms of periodontal disease.

1.1.2 Case definitions for periodontitis

Currently there is a lack of uniformity in the definition of periodontitis for the purposes of epidemiological and other research studies and specifically there is no universal consensus to define a case of chronic or aggressive periodontitis. The adoption of universal case definitions for periodontal disease is of great importance in the research field. A wide range of different case definitions for periodontitis have been described and used in different studies, resulting in considerable confusion and difficulty in comparing studies directly. For instance, the descriptions of disease prevalence in epidemiological studies need an obvious and a clear disease definition, which can easily applied to the population cohort. Due to the fact that the criteria used to identify periodontal disease tend to be continuous variables (e.g. PPD & CAL) as well as the disease is site specific, this case definition is preferred to be dichotomised.

The use of different case definitions has a great impact on the description of prevalence, severity and extent of periodontal disease. PPD and CAL are the most commonly used clinical parameters to measure the presence, distribution and severity of periodontal disease. Therefore, these continuous variables require a clear threshold in order to establish periodontal disease. Although CAL is a robust measure of the severity of periodontal disease, loss of attachment can occur without presence of periodontitis like in case of overhang restorations (Page and Eke, 2007). More recently, increasingly the case definitions described below have attracted some consensus for use in future studies where these definitions include both PPD and CAL in order to capture a true case of periodontitis.

The Centre for Disease Control and prevention/ American Academy of Periodontology (CDC/AAP) has suggested the following criteria for a case with moderate or severe chronic periodontitis (Page and Eke, 2007):

a) Moderate Periodontitis: Patients with two or more interproximal sites with clinical attachment loss of ≥4mm occurring at two or more different teeth OR Two or more interproximal sites with a probing depth of ≥5mm, not on the same tooth.

b) Severe Periodontitis**:** Patients with two or more interproximal sites with clinical attachment loss of ≥6mm, not on the same tooth, and the presence of one or more interproximal sites with a probing depth of ≥5mm.

Demmer and Papapanou have discussed the difficulties of clinical diagnosis of aggressive periodontitis using the published features of this condition. They have suggested the following criteria in order to diagnose a patient with Aggressive Periodontitis for research purposes (Demmer and Papapanou, 2010) which they base on the suggestion that this severity of destruction would not be commensurate with the amount of plaque present irrespective of plaque control:

a) In patients ≤25 years of age, the presence of two or more interproximal, nonadjacent sites with attachment loss of ≥4 mm occurring at a minimum of two different teeth and accompanied by bleeding on probing, will signify aggressive periodontitis.

b) In individuals between 26 and 35 years of age, a diagnosis of aggressive periodontitis will require the presence of two or more interproximal, nonadjacent sites with attachment loss of ≥6 mm occurring at a minimum of two different teeth and accompanied by bleeding on probing.

1.1.3 Treatment of Periodontal Diseases

The major objectives of periodontal therapy are the control of periodontal infections and the maintenance of functional teeth for a lifetime. In general, there is a consistent chronological order to the management of plaque associated periodontal disease.

Mechanical non-surgical periodontal therapy, which includes oral hygiene instruction, supragingival scaling & root surface debridement, is considered to be highly effective for the treatment of periodontitis (Badersten et al., 1985; Hill et al., 1981; Lindhe et al., 1982; Ramfjord et al., 1987). Despite the generally good response to this therapy, studies have shown variations in treatment responses between patients and that some patients and sites did not respond to therapy (Claffey et al., 1996; Egelberg and Claffey, 1994; Hirschfeld and Wasserman, 1978).

Most of these studies have been carried out on patients with chronic periodontitis. The management of aggressive periodontitis may be more challenging as many studies recommend the use of antimicrobials as an adjunctive to the mechanical therapy (Haffajee et al., 2003; Herrera et al., 2002; Hung and Douglass, 2002; Kamma and Baehni, 2003; Sigusch et al., 2001). However, the evidence for the use of adjunctive antimicrobials is still equivocal. For example in another study for the treatment of generalised aggressive periodontitis, the use of mechanical debridement alone showed 54% reduction in pocket depth, whereas the additional use of the combination of amoxicillin and metronidazole increased this figure to 74% (Guerrero et al., 2005).

While the merits of non-surgical therapy are not in dispute the decision to proceed to surgical therapy is not always straightforward. In a study where patients were treated by non-surgical and surgical techniques and monitored over 5 years, they showed that both modalities were effective in establishing clinically healthy gingiva and preventing further attachment loss (Lindhe et al., 1982). They suggested in a later study that the critical determinant was not the technique but the quality of the debridement that was crucial (Eaton et al., 1985; Lindhe et al., 1984).

Eaton and co- workers demonstrated that root planing under direct vision at the time of surgery was more effective than blind instrumentation in removing subgingival deposits (Eaton et al., 1985). However, in no instance was any root surface found to be completely free of deposits. On the other hand, performing periodontal surgery on plaque-infected dentitions led to recurrence of the disease including significant attachment loss (Nyman et al., 1977). However, Pihlstrom and co-workers reviewed the literature and concluded that both surgical and non-surgical therapy resulted in probing pocket depth reduction with neither procedure being uniformly superior. In addition, tooth retention appeared to be more related to susceptibility or case type than the method of therapy performed (Pihlstrom et al., 1983).

Irrespective of the type of treatment provided, a site which is responsive to treatment will show resolution of gingival inflammation (no redness, no swelling, and no bleeding on probing), reduction of pocket depths, and gain of clinical attachment level. However these outcomes may be considered as surrogate endpoints of treatment, and tooth survival/loss is a" true" end point of periodontal treatment outcome. There are not many studies that have looked at the prevalence of tooth loss both during active periodontal therapy and during long-term maintenance. Those that have report an overall percentage of tooth loss of 7.8% (Konig et al., 2002), 8.3% (Carnevale et al., 2007) and 8.8% (Tonetti et al., 2000). Even teeth with advanced periodontal disease can be kept healthy in the long term if a strict periodontal maintenance is applied. A 14 year longitudinal study of treating and maintaining patients with advanced periodontal diseases showed that only 2.3% tooth loss may occur (Lindhe and Nyman, 1984).

Interestingly, a systematic review concerned with tooth loss during long-term periodontal maintenance (≥5 years) concluded that long-term periodontal maintenance maintained periodontal health and prevented tooth loss in most patients. These findings led to low rates of tooth loss due to periodontal reasons (Chambrone et al., 2010). This includes the finding of a 30-year maintenance study by Axelsson and co-workers that showed the prevalence of periodontal disease progression and tooth loss was very small (Axelsson et al., 2004). This was particularly the case when patients were under a strict maintenance programme (Checchi et al., 2002). In addition, the periodontal stability of patients maintained by a specialist was much better than ones maintained by general dentist (Eickholz et al., 2008; Fardal, 2006).

Overall, treatment failures tended to cluster in a small cohort of the treated patients, and partially associated with periodontal maintenance and with the adverse effects of smoking. These observations also suggest the involvement of other factors may also affect treatment outcomes, and these might include other recognised periodontal risk factors such as genetics, systemic health, and type of bacteria involved.

1.1.4 Prognostic Factors of Periodontal Disease

Prognosis is the prediction of the course, duration and outcome of a disease based on the pathogenesis and presence of risk factors for the disease. Usually prognosis is estimated after establishing diagnosis and before planning treatment.

Development of an accurate prognosis is an essential part of treatment planning in periodontal clinics. Different practitioners may assign varying prognoses for the same tooth, as there are no well-established universal guidelines for assignment of periodontal prognosis. Dental practitioners usually rely on the clinical parameters to predict the long term outcome of the provided periodontal therapy. The most used measures are Probing Pocket Depth (PPD), Gingival Recession & Attachment Level (AL). Other clinical parameters have also been used to assess the prognosis such as bleeding on probing (BoP), presence of plaque, furcation involvements and the site of the lesion (Persson, 2005). However, previous studies suggest that these clinical parameters are relatively poor prognostic factors of future disease progression especially in the short term (Claffey, 1991; Claffey etal.,1990; Haffajee et al.,1988).

Although BoP is an essential criterion for the diagnosis of gingivitis and an indicator of periodontal disease activity, it is limited as a periodontal prognostic indicator (Armitage, 1996; Lang et al., 1986). Furthermore, Claffey and Egelberg concluded in their study that residual probing depth was moderately predictive of further disease progression, whereas persisting bleeding on probing was not (Claffey and Egelberg, 1995).

The traditional paradigm that is used to assign teeth prognoses is based on an outdated model of disease progression that assumes all subjects are equally susceptible to gum disease and that all plaque is the same. Under this paradigm the most commonly used clinical parameters in assigning prognosis can be divided into two categories (McGuire and Nunn, 1996) Table 1-3:

1) Individual tooth prognosis & 2) Overall prognosis

Individual tooth prognosis	Overall prognosis
Percentage bone loss	Age
Probing depth	Medical status (smoker &/or diabetic)
Distribution and type of bone loss	Rate of progression
Presence and severity of furcation	Patient cooperation
Mobility	Economic considerations
Crown/Root ratio	Knowledge and ability of dentist
Root form	Family history of periodontal diseases (mother, father, siblings)
Caries/Pulpal involvement	Oral hygiene (good, fair, poor)
Tooth position and occlusal relationship	Maintenance interval
Fixed or removable abutment	Para-functional habits (± bite-guard)

Table 1-3:The commonly taught clinical factors used in assigning prognosis (McGuire and Nunn, 1996).

One study made a comparison between prognosis and actual outcome in order to determine if the widely used clinical parameters are effective in assigning an accurate individual tooth prognosis (McGuire and Nunn, 1996); the results indicate that teeth with good prognosis generally remained good. While in teeth with less than good prognosis the overall accuracy in assigning prognosis was only 43% at 5 years and 35% at 8 years. Furthermore, in a later study (McGuire and Nunn, 1999) they concluded that heavy smoking and positive IL-1 genotype are significantly associated with tooth loss.

As periodontal disease is site-specific, it may be difficult to assess accurately the overall treatment outcome from different sites within the same individual. In a study by D'Aiuto and co-workers of prognostic factors, the authors suggested that site-specific factors were the major determinants of initial outcome of treatment in severe periodontitis (D'Aiuto et al., 2005c). However, this would tend to be contradicted by the widely noted observation that treatment failures (i.e. tooth loss) tend to cluster in a relatively small group of treated patients (Chambrone et al., 2010).

In general, systemic or patient level prognostic factors need to be considered, such as smoking, diabetes or genetic factors, as well as local factors such as furcation or mobility. Regarding the relationship between the commonly taught clinical parameters and tooth loss rate, some clinical factors, such as satisfactory crown/root ratio, heavy smoking, mobility or furcation involvement, contributed significantly to predict the tooth loss rate, while other factors, such as patient age or root form, showed a weak relationship to the probability of tooth loss (Nunn et al., 2012).

In a longitudinal prospective study over 30 years of professional periodontal maintenance of patients with periodontal disease, the incidence of caries, periodontal disease progression and tooth loss was very low (Axelsson et al., 2004). In addition, in a later study Eickholz and co-workers identified that irregular participation in supportive periodontal treatment was the strongest influence associated with tooth loss over other factors like age, sex, baseline diagnosis, IL-1 polymorphism and smoking (Eickholz et al., 2008).

Both clinical and certain immunological and microbiological parameters have also been evaluated for predicting change in clinical status and tooth loss (Machtei et al., 1997; Machtei et al., 1999). Mean probing depth, mean attachment loss, crestal bone height, baseline smoking status, and cotinine level were all associated with bone and attachment loss over time. The presence of *Bacteroides forsythus, Prevotella intermedia* and *Porphyromonas gingivalis* was linked to future periodontal breakdown (Machtei et al., 1997). Loss of crestal bone height, baseline attachment loss, and different systemic conditions were associated with increased tooth loss over time, while the presence of *B.forsythus* doubled the risk of tooth loss over time (Machtei et al., 1999).

Therefore, in view of the difficulties in accurately assessing prognosis, there is increased interest in improving the way of assessing the individual patient clinically for diagnostic and prognostic purposes.

1.1.5 Biomarkers and Periodontal Diseases

Periodontal disease is irreversible, multifactorial and cumulative in nature and both dental plaque and host factors are involved in determining its progression. Therefore, there is no single way either clinically or by laboratory testing that can describe the true pathogenesis of periodontal disease. This leads to the thinking of other novel ways such as identifying and detecting host and bacteria-derived proteins as surrogate periodontal biomarkers. These proteins (markers) can be obtained from sampling saliva, serum, GCF, sub-gingival plaque and gingival tissue.

The current diagnosis of periodontal diseases is based on visual and radiographic examinations (which has a lot of subjectivity), which often indicate only the consequences of past diseases. Early prediction of risk and accurate diagnosis of current disease activity are needed for effective prevention and treatment. That is why periodontists would greatly value an objective diagnostic tool that can provide reliable information to assess the existence, severity and outcome of disease (Buduneli and Kinane, 2011; Sexton et al., 2011).

The term “biological marker” has been defined as “an indicator that signals events in biological systems or samples, and it is generally taken to be any biochemical, genetic or immunologic indicator that can be measured in a biological specimen” (Hulka and Wilcosky, 1988; Schulte, 1989).The biomarker can be extrinsic or intrinsic, diagnostic or prognostic, and can be subdivided into many categories.

In periodontology there is thus a need for a marker that is able to distinguish accurately between different disease categories, ideally with both high sensitivity and specificity. i.e. a test with great diagnostic potential. In this context sensitivity is defined as the ability of a diagnostic test to identify the disease when it truly exists. Specificity is the probability of a diagnostic test being negative when the disease is truly absent. Interestingly, demonstration that average concentrations of biomarker are different between two groups (disease categories) does not always indicate that this marker has a true diagnostic or prognostic utility.

Oral fluids usually contain large amounts of serum proteins, inflammatory mediators, cell degradation products, microbial metabolites and enzymes (Kinney

et al., 2007; Sorsa et al., 2004a; Uitto et al., 2003), suggesting they may be a very useful and accessible source of biomarkers for a wide range of conditions. In particular, saliva and gingival crevicular fluid (GCF) have been investigated extensively as these oral fluids may reflect the disease status of the oral cavity (Kaufman and Lamster, 2002; Lamster and Ahlo, 2007; Taba et al., 2005).

The identification of biological markers that are associated with periodontal disease activity might help in the development of new periodontal therapies, in detection and monitoring of disease, in stratifying patients for diagnostic, prognostic and treatment planning purposes, and as surrogate measures of periodontal disease in field studies of periodontal disease where full periodontal examination might be impractical.

Although a wide range of host- and pathogen-derived markers have been investigated in these body fluids, there is currently no one single biomarker with sufficient power to confidently detect or predict the presence of periodontal disease (Kaufman and Lamster, 2000; Ozmeric, 2004). Broadly, potential periodontal disease markers can be classified into four main groups (Chapple, 1997) that indicate:

1. Presence of putative periodontal pathogens.
2. Gingival and periodontal inflammation.
3. Host`s immune response.
4. Host tissue destruction.

Due to the complex nature of periodontal disease, it is more likely that multiple, and not individual, biomarkers together can discriminate between health and disease or disease onset and progression, enhance the outcome disease prognosis, prioritise patients according to their treatment need and identify the ones who will respond well to the offered treatment.

In addition, consideration has to be made as to the source fluid of the biomarker, which might be gingival crevicular fluid, saliva or blood.

Gingival Crevicular Fluid (GCF)

GCF is a serum transudate or inflammatory exudate that seeps into gingival crevices or periodontal pockets around teeth with inflamed gingivae, it is composed of serum and locally generated materials such as tissue breakdown products, inflammatory mediators, and antibodies directed against dental plaque bacteria (Armitage, 2004a).

GCF acts as a medium for the transport of bacterial products into and host derived products out of the periodontal environment. The interest in the diagnostic potential of GCF has increased since the suggestion by Brill that analysis of the GCF might be a way to evaluate quantitatively the inflammatory status of gingival and periodontal tissues (Brill, 1962). GCF has been suggested by some researchers to be the most promising medium for the collection of diagnostic information, due to its location adjacent to periodontal tissue where periodontal disease starts (Chapple, 1997).

Bacterial antigens trigger monocytes to release inflammatory mediators including $PGE_{2,}$ IL-1, IL-6, IL-8, TNF and collagenase that increase local destruction of the connective tissues. Therefore, levels of these monocytic inflammatory mediators may well represent the site level markers of disease activity. In addition, elevated levels of GCF neutrophil mediators including neutrophil elastase, β-glucoronidase and LTB_4 may reflect acute episodes of localised tissue destruction (Champagne et al., 2003). Over 65 GCF markers have been examined as possible markers for periodontal disease progression (Armitage, 2004a; Ozmeric, 2004)

Table 1-4.

In a review by Loos and Tjoa, the authors concluded that there was no single or combination of GCF markers available to use in order to distinguish between chronic or aggressive periodontitis, as well as to predict the outcome of periodontal treatment (Loos and Tjoa, 2005). Therefore, well-controlled longitudinal clinical trials are needed in order to establish reliable biomarkers for periodontal disease as most published work about biomarkers and periodontal disease based on cross-sectional studies.

Enzymes	Proteins	Igs	Cytokines	Others
Lysozyme	Lactoferrin	IgA	VEGF	PAF
MMP-8 (collagenase 2)	cystatinsC,S	IgG	IL-1β	leukotriene B4
MMP-2	Neopterin	IgM	TNF-α	thromboxane B2
MMP-9	β-NAH	IgE	IL-2	hydroxyproline
MMP-13 (collagenase-3)	TIMP		INF-α	lipoxin A
Neutral protease	osteopontin		IL-10	keratin
Dipeptidylpeptidase	calprotectin		RANTES	substance P
Alkaline phosphatase	hyaluronic acid		IL-8	PGE2
Aspartate aminotransferase	chondroitin sulphate		IL-1ra	glucose
Myeloperoxidase	Endothelin		IL-4	ICAM-1
Creatine kinase	proteoglycan		IL-6	methylglyoxal
Lactate dehydrogenase	thrombomodulin		TGF-β	lactic acid
Elastase	transferrin		HGF	propionic acid
β-Glucuronidase	C-reactive protein		EGF	butyric acid
Cathepsin G,D, B	α-2-macroglobulin			phylloquinone
Plasminogen	α-1-antitrypsin			Volatile sulphur compounds
Gingipain	Osteocalcin			glutathione
	osteonectin			hydroxylysylpyridinoline
	Hyaluronan			
	Fibronectin			
	ICTP			
	α-1-EPI			
	NTx			
	E-selectin			
	neurokinin-A			
	MRP-8			
	calcitonin			
	albumin			

Table 1-4: Possible GCF markers for periodontal disease suggested for potential diagnostic or prognostic use. Acknowledgement (Ozmeric, 2004).

Saliva

On a practical level, salivary analysis offers significant advantages when used for diagnostic purposes, as it is easily collected in a non-invasive way. In addition, it may provide a feasible, cost-effective approach for screening a large number of patients (Lamster and Ahlo, 2007). Furthermore it is most likely that salivary biomarkers would be most suited to assessing patient-level diagnostic or prognostic biomarkers as it is not likely to reflect site specific differences.

Whole (mixed) saliva is a combination of oral fluids that originates from secretions of the minor and three paired major salivary glands, bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial by-products, viruses, fungi, desquamated epithelial cells, food, cellular components and GCF (Kaufman and Lamster, 2000; Mandel and Wotman, 1975; Sreebny, 1988) Figure 1-1. Therefore, whole saliva represents a complex balance between local and systemic sources, and biomarker analysis of such a pooled sample might provide an overall periodontal disease assessment rather than site-specific GCF analysis. This is why saliva has great potential in the diagnosis of oral and systemic conditions (Good et al., 2007; Lee et al., 2009; Malamud, 2011). It has been shown that out of 2290 identified WS proteins, there are 1163 specific for WS which are neither found in plasma nor in glandular saliva (Loo et al., 2010) Figure 1-2.

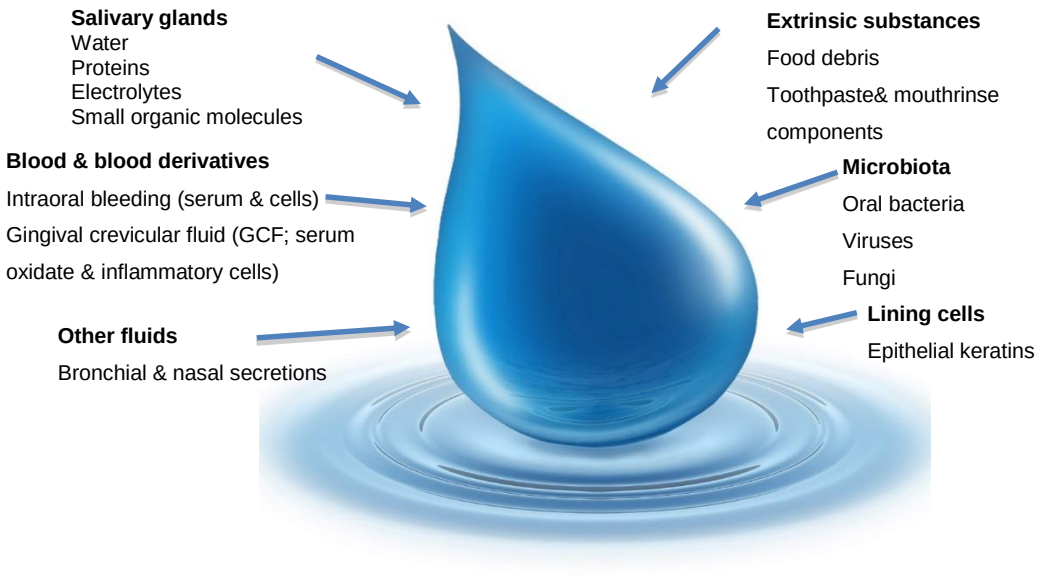

Figure 1-1: Components of whole saliva (WS). Acknowledgement: (Kaufman and Lamster, 2000).

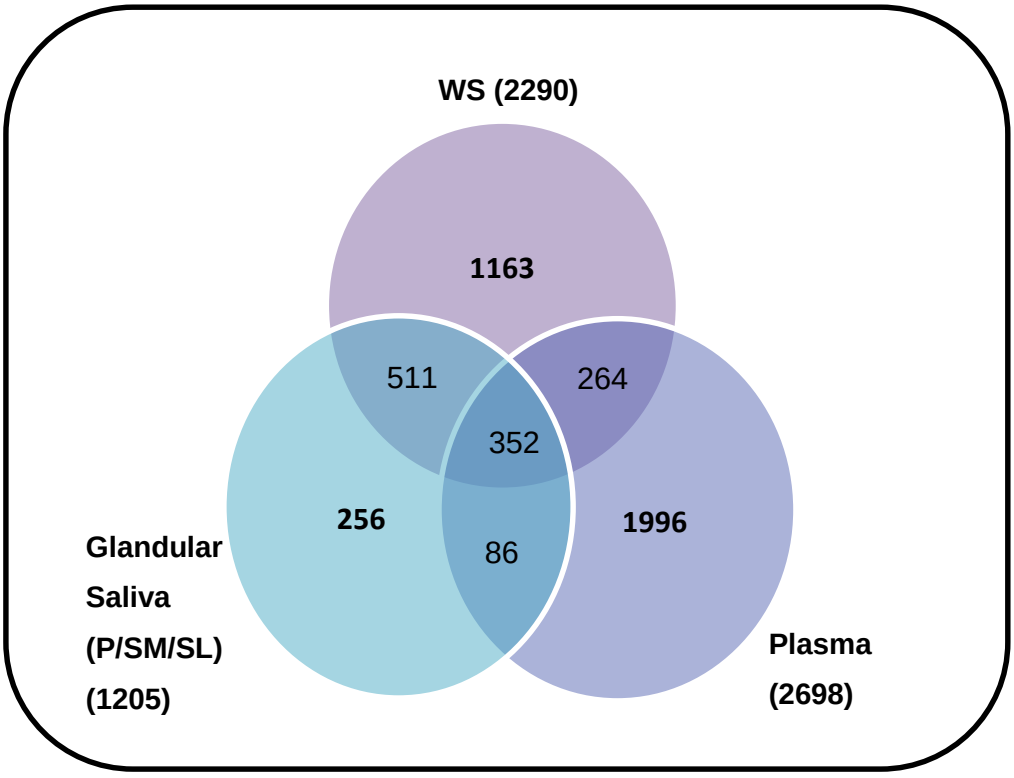

Figure 1-2: Venn diagram showing the overlapping protein identifications among plasma, whole saliva, and parotid/SM/SL. Acknowledgement (Loo et al., 2010).

Salivary biomarkers

As mentioned earlier, whole saliva contains more than two thousand proteins (Loo et al., 2010). Out of these proteins, in the study here we were particularly interested in four proteins as potential periodontal biomarkers, specifically Matrix Metalloproteinase-8 (MMP-8) and the antimicrobial peptides (AMPs) Human Neutrophil Peptide 1- 3 (HNP1-3), S100A8 and LL-37. The reason for choosing MMP-8 was due to the wealth of research indicating that MMP-8 is a promising biomarker for periodontal disease (Miller et al., 2006; Todorovic et al., 2006). For the AMPs HNP1-3, S100A8 & LL-37 results from a recent study in our laboratory have identified these three AMPs, using mass spectrometry techniques, as potential diagnostic biomarkers for periodontal disease (Mulli, 2012). In this study Dr.T.Mulli was able to identify 67 proteins out of 115 in saliva and GCF using Surface Enhanced Laser Desorption Ionization- Time Of Flight Mass Spectrometry (SELDI-TOF MS) with the potential to distinguish between gingivitis and periodontitis. Only 3 distinct peaks on the SELDI MS (HNP1-3, LL-37& S100A8) were conclusively identified using Liquid Chromatography quadruple Mass Spectrometry (LC-MS/MS). Interestingly, these are all known antimicrobial peptides (AMPs) and therefore of considerable potential biological significance in periodontal disease. In addition, he showed the potential diagnostic utility of these biomarkers, particularly HNP1-3 & S100A8. However he also found that the method used for saliva collection had marked effects on the concentration of some salivary analytes. He used salivettes & spitting methods to collect saliva samples in different studies and the spitting method showed much higher concentrations of analytes (AMPs) than salivettes. Because of this he was unable to validate specified cut-off points of AMP concentrations in a separate population of samples as they were collected using different methods.

MMP-8 (Matrix Metalloproteinase-8) (Neutrophil Collagenase II) (Collagen Cleaving Enzyme) (Neutrophil-derived proteolytic enzyme):

Proteins of the matrix metalloproteinase (MMP) family consist of approximately 25 members that can be categorised into five main groups: Collagenase, Gelatinase, Stromelysins, membrane-type, and others. They are involved in the breakdown of collagen and extracellular matrix (ECM) in normal physiological processes, such as embryonic development, reproduction, and tissue remodelling, as well as in pathological processes, such as arthritis and metastasis (Sorsa et al., 2004). They may play an important role in the pathogenesis of periodontal disease, in which their role should not be considered as destructive factors only but which may also have protective or defensive roles (Sorsa et al., 2011; Sorsa et al., 2004). For example physiological levels of MMP-8 can perform an anti-inflammatory effect by processing some cytokines and chemokines (Sapna et al., 2014). Most MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. MMP activity is also regulated by Tissue Inhibitors of MetalloProteinases (TIMPs) that are responsible for protecting the PDL from degradation by MMPs in the state of health. TIMPs can play a role in MMPs stabilisation and transportation alongside their major role as MMPs inhibitors (Sorsa et al., 2006). Degradation of ECM, collagen fibres and alveolar bone might be triggered by the imbalance between MMPs and their inhibitors (Reynolds, 1996).

The gene encoding MMP-8 is called *homo sapiens complex locus MMP8*, and the enzyme encoded by this gene is stored in secondary granules within neutrophils and is activated by autolytic cleavage. Its function is degradation of type I and III collagens, which is critical for periodontal tissue destruction in periodontitis but not for physiological gingival tissue remodelling (Rai et al., 2008). The gene is part of a cluster of MMP genes which localize to chromosome 11q22.3 (NCBI website).

It is found in specific granules in neutrophils, but is also expressed by diverse cell types including epithelial cells, fibroblasts, macrophages and endothelial cells (Van Lint and Libert, 2006). It exists in the inflammatory exudate within the gingiva and in oral fluids (saliva & GCF) due to the permeability of the sulcular epithelium (Miller et al., 2006).

In addition to MMP-8, there are other types of MMPs that are involved in periodontal tissue destruction including MMP-9, MMP-13 (Hernandez et al., 2006; Hernández Ríos et al., 2009), MMP-25 & MMP-26 (Emingil et al., 2006) which all have been suggested as potentially useful biomarkers for progression of periodontal disease. Also, the gelatinase group (MMP-2 & MMP-9) may participate in periodontal tissue destruction during periodontitis (Makela et al., 1994), and their levels have been shown to reduce after periodontal therapy (Gursoy et al., 2013; Marcaccini et al., 2010). However, MMP-8 is the most studied MMP in relation to periodontal disease and particularly as a potential disease biomarker.

In previous studies MMP-8 levels in patients with periodontal disease were an indicator for disease severity as well as activity (Herr et al., 2007; Romanelli et al., 1999). A recent study by (Adina Bianca Boşca, 2012) concluded that MMP-8 may be a reliable diagnostic marker of periodontal disease, as it was significantly higher in patients with chronic periodontitis compared to healthy controls. Also, they showed that there was a correlation between raised levels of MMP-8 and the severity of periodontitis assessed clinically & histologically. Furthermore, MMP-8 has been suggested as a potential periodontal biomarker between health and disease (Miller et al., 2006; Todorovic et al., 2006), as well as a prognostic marker of periodontal disease (Passoja et al., 2008; Ramseier et al., 2009).

In studies investigating the relationship between Salivary and GCF MMP-8 levels and periodontal disease, MMP-8 levels were significantly higher in subjects with chronic periodontitis than subjects with gingivitis and healthy controls (Rai et al., 2008; Xu et al., 2008). Also, it has been suggested that persistent elevation of GCF MMP-8 levels might be indicative of high risk to periodontal disease and poor response to periodontal treatment (Mäntylä et al., 2006). Moreover, Kinane and co-workers in their intervention study included 20 subjects with chronic periodontitis, concluded that the MMP-8 GCF levels were reduced significantly following periodontal treatment (Kinane et al., 2003). A further treatment study investigated the MMP-8 plasma levels of 28 patients with periodontitis and 22 controls and concluded that MMP-8 levels were higher in the diseased group but reduced significantly 3 months after non-surgical periodontal treatment (Marcaccini et al., 2009b).

Mantyla and co-workers have developed a chair-side GCF MMP-8 test (Lab on Chip (LOC)) to differentiate periodontitis from gingivitis or healthy sites, as well as

to monitor treatment outcome of periodontitis. This is a potentially useful first MMP-based point-of-care (POC) test developed to diagnose a tissue destructive oral disease (Mäntylä et al., 2003). Also, Herr and co-workers have introduced a microfluidic assay for POC rapid quantification of salivary MMP-8 to identify subjects with periodontitis (Herr et al., 2007). In addition, the MMP-8 oral rinse sample analysis POC test could be clinically beneficial in rough screening to identify subjects who at risk to develop periodontitis, as well as to define the maintenance phase visits after active periodontal treatment (Leppilahti et al., 2010).

A recent study by Gursoy and co-workers concluded that MMP-8 is a strong biomarker candidate for detecting alveolar bone destruction, as their levels were higher in subjects with severe bone loss than those with slight bone loss (Gursoy et al., 2013).

Antimicrobial Peptides (AMPs)

Antimicrobial peptides are defined as peptides that are smaller than 100 amino acids, usually 12-50 amino acids, which act like broad-spectrum natural antibiotics and have the ability to kill different species/ strains of oral bacteria. In addition, AMPs have antiviral, antifungal and antiparasitic activities. Therefore, AMPs play an important role as part of the innate host defence mechanisms against microbial colonization both synergistically and independently (Diamond et al., 2009). Only 45 AMPs have been identified in saliva and many of them do exist in GCF as well (Gorr and Abdolhosseini, 2011). Examples of these AMPs families are: defensins, cathelicidins and histatins. See Figure 1-3 for the summary of AMPs' functions.

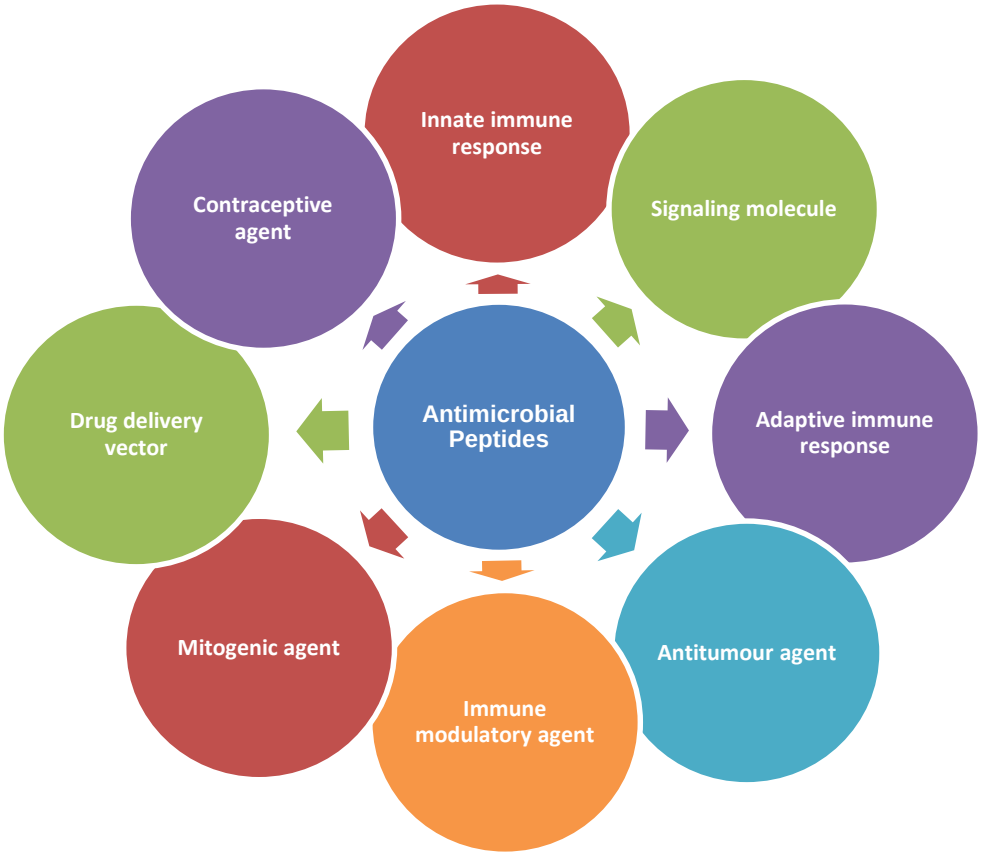

Figure 1-3: Schematic draw showing the multifunctional properties of antimicrobial peptides. Acknowledgement (Pushpanathan et al., 2013).

Most AMPs are cationic peptides (positively charged) and have both hydrophilic and hydrophobic sides. Therefore, they are attracted to the anionic (negatively charged) bacterial membranes, dissolve in aqueous and are stable in lipid-rich

environments (Brogden, 2005). The most accepted hypotheses of their action are the disruption of microbial membrane function by forming pores using one of three means (Brandenburg et al., 2012) Figure 1-4:

A. Barrel-stave: in which the peptides aggregate and penetrate the bacterial bilayer membrane to align the hydrophobic regions of the peptides with the bacterial lipid core region, and the hydrophilic peptide regions form the pore core.
B. Toroidal: in which the peptides aggregate and cause the lipid monolayer to bend continuously through the pore, then the water core is lined by both the inserted peptides and the lipid head groups.
C. Carpet channels: in which the peptides disrupt the lipid bilayer membrane by aligning parallel to it and form a carpet.

Overall, the final effect of all these means is that some monomer can pass to the cytoplasm and bind to the cellular DNA and RNA, causing protein synthesis inhibition or protein folding. In addition, increasing cell permeability and causing cell death (Brandenburg et al., 2012).

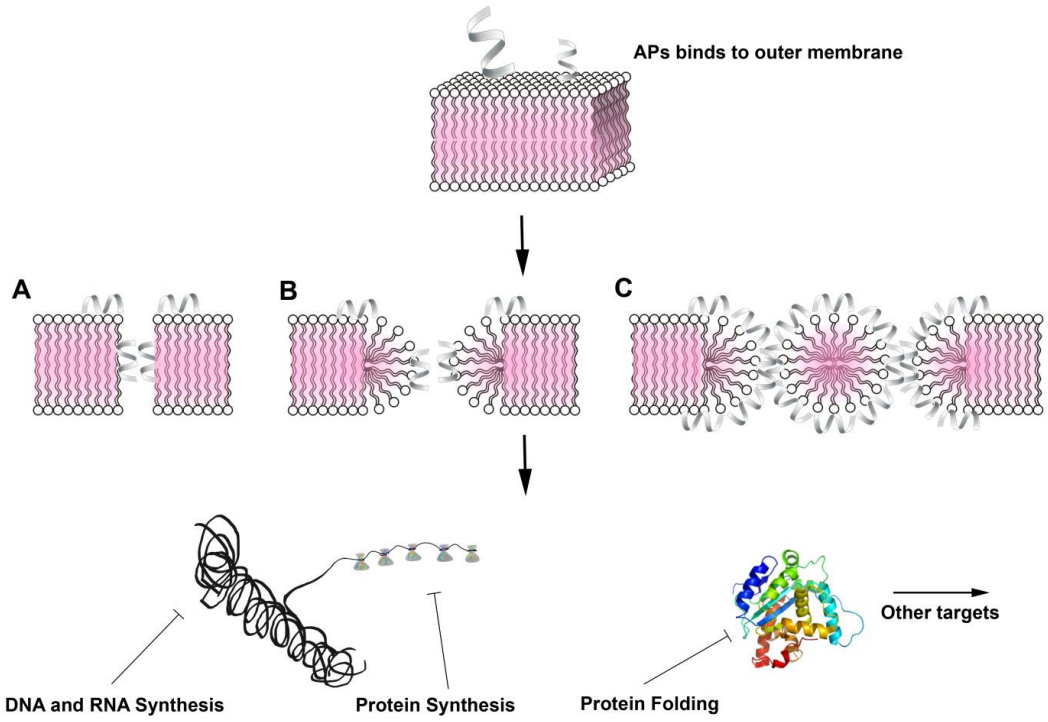

Figure 1-4: Schematic draw showing the proposed AMPs mechanism of action in bacteria. Acknowledgement (Brandenburg et al., 2012).

Interestingly, it has been suggested that the high risk of periodontal disease in patients with systemic diseases might be attributed to the alteration in these

salivary AMPs. For example patients with diabetes reportedly have low concentrations of statherin and Histatin 1 & 3, and high concentrations of HNP1, 2, 4 and S100A9 compared to healthy controls (Cabras et al., 2010). Moreover, Morbus Kostmann disease in humans is due to deficiency in HNP1-3 and LL-37, in which patients suffer from severe periodontal disease and recurrent oral infections (Putsep et al., 2002).

HNP1-3 (Human Neutrophil Peptides) (α-defensins)

These AMPs are three of the six different human α-defensins. Neutrophils are the main source of these antimicrobial peptides, where they are stored in azurophilic (primary) granules in a biologically active form (Ganz, 2003; Ganz et al., 1985). However, they can be released from other cell types including monocytes, B & T-lymphocytes (Schneider et al., 2005). Surprisingly, the number of neutrophils is unrelated to the levels of HNP1-3 in patients with periodontal disease which emphasises the fact that HNP1-3 can be secreted from cells other than neutrophils, or alternatively points to complexities in the regulation of their production and secretion (Puklo et al., 2008).

Alpha defensins are not completely active within the PD pockets. They have a wide range of antimicrobial activity as well as a stimulatory effect on fibroblast proliferation. Their bactericidal activity can be aborted, by proteolytic degradation or by the action of dermatan sulphate containing proteoglycan that is released by bacteria proteases (Schmidtchen et al., 2001).

It has been previously reported that the concentration of GCF HNP1-3 is much higher in patients with chronic periodontitis than those with aggressive periodontitis and very low in healthy controls (Puklo et al., 2008). In contrast other studies have not shown any significant differences in the amount of α-defensins between subjects with healthy and diseased periodontium (Lundy et al., 2005; Turkoglu et al., 2010).

As well as their potential protective effects, elevated local levels of α-defensins in periodontal tissues may also have destructive effects. HNP1–3, at concentrations of 5–8µg/ml, stimulate the proliferation of periodontal epithelial cells, and at very high concentrations (of 50µg/ml) induce cell-death of human oral fibroblasts (Nishimura et al., 2004).

S100A8/MRP8 (Myeloid Related Protein 8) (Calgranulin A)

This peptide is one of the twenty members of low molecular weight calcium binding proteins, S100 family. It forms a dimer with S100A9/MRP14 (Calgranulin B) called Calprotectin, which is a crucial pro-inflammatory mediator that is highly expressed in inflammatory sites by neutrophils and monocytes (Kido et al., 1999; Sun et al., 2011). It plays an important role in innate immune mechanisms as it induces neutrophil chemotaxis and adhesion (Sun et al., 2011), and shows antimicrobial activity by its zinc-chelating action (Gorr and Abdolhosseini, 2011).

Some studies have documented that patients with diabetes, cardiovascular disease and prostate cancer have high levels of calprotectin (Altwegg et al., 2007; Bouma et al., 2004; Hermani et al., 2005). Moreover, GCF calprotectin levels of patients with periodontal disease were higher in diseased sites than healthy ones (Kido et al., 1998; Li et al., 1998; Zhou et al., 1998). Similar results have been found in plasma calprotectin levels in patients with aggressive periodontitis (Sun et al., 2011). Therefore, these findings may provide more support to the association of periodontal disease with systemic disease. However in another study by (Haigh et al., 2010) it was concluded that S100A8 & A9 are increased after treatment of periodontal disease.

LL-37 (Cathelicidin)

LL-37 is the only cathelicidin-derived AMP in humans, where it can be found in most body fluids and shows a potent and broad spectrum antimicrobial activity (Nijnik and Hancock, 2009). Like α-defensins neutrophils are the most abundant, but not the only, source of LL-37, where it is synthesized as preproproteins and stored within specific (secondary) granules in an inactive form. Although saliva can partially inhibit the activity of LL-37, it protects the peptide from degradation by gingipain protease secreted by dental plaque bacteria (Gutner et al., 2009).

LL-37 may play an important role in protecting the human periodontium from dental plaque bacteria, and has been reported to be especially effective against *A.a* (Gomez-Garces et al., 1994). This supports the findings of a later study by Puklo and co-workers that local deficiency of LL-37 can be found in severe cases of periodontitis (Puklo et al., 2008). On the other hand, another study found that

LL-37 GCF levels were significantly higher in patients with chronic periodontitis (Türkoğlu et al., 2009). An interesting finding by Nagaoka and co-workers is that LL-37 is crucial for the efficacy of the α-defensins' antimicrobial activity as both AMPs work synergistically (Nagaoka et al., 2000).

Serum

Blood is by far the most popular and accepted choice for biomarker testing clinically in medicine, due to the fact that blood is the circulating fluid that surrounds all tissue and organs and collects by-products from diseased areas. Periodontal disease is inflammatory in nature and can stimulate the body to release inflammatory cytokines such as IL-1β, IL-6 & TNF-α not only locally, but also systemically (Offenbacher et al., 1996). These cytokines may cause chronic low-grade systemic effects that involve hepatic secretion of CRP (Scannapieco, 2004) Figure 1-5.

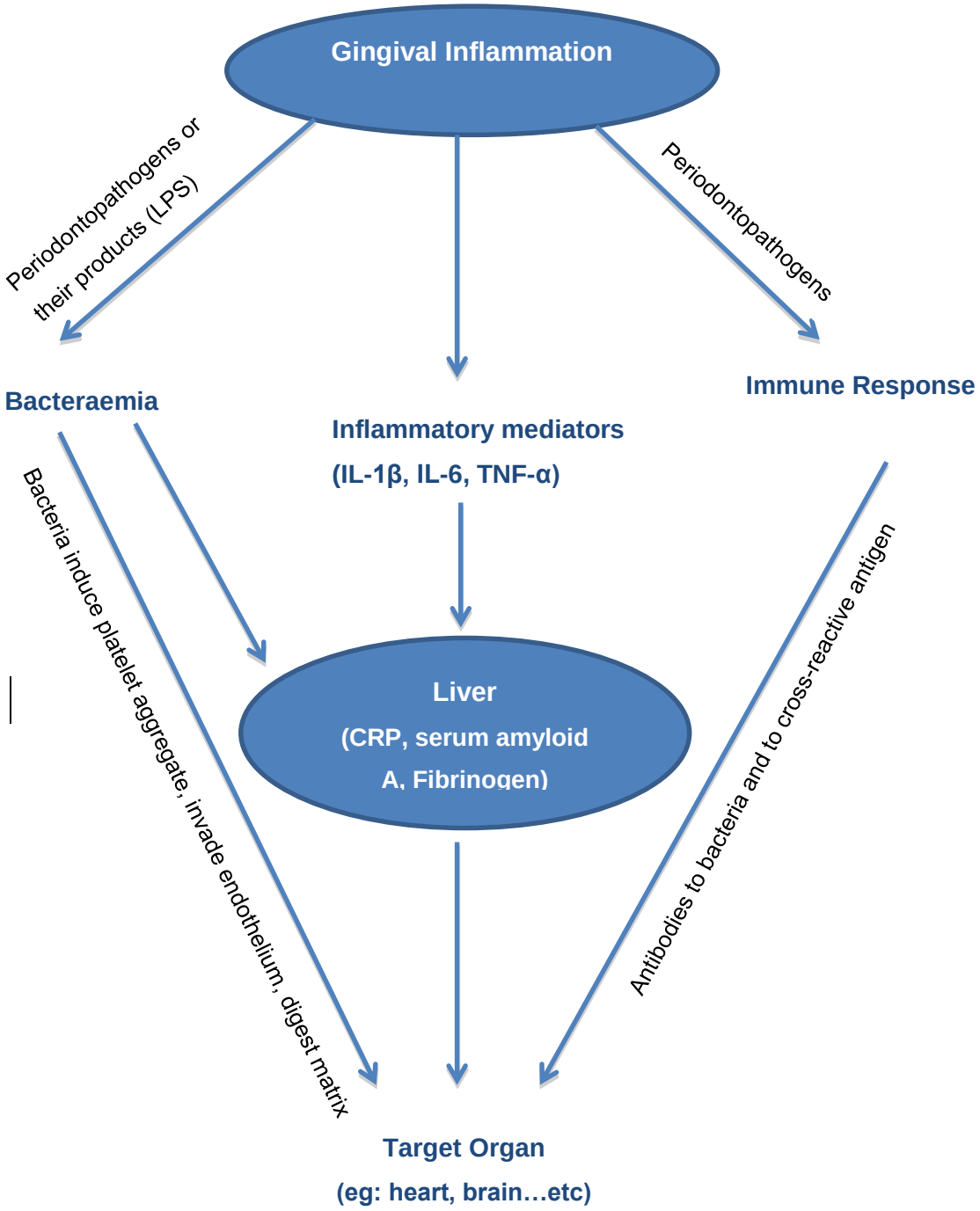

Figure 1-5: Theoretical mechanisms by which gingival inflammation may modulate systemic disease (Acknowledgement (Scannapieco, 2004)**).**

Therefore, it has been proposed that periodontitis may play a role in the aetiological mechanisms of many systemic diseases via this induction of systemic inflammation. For example: IL-6 & CRP are considered reliable markers to

measure the inflammatory level of an individual and their levels are elevated in subjects with severe periodontitis (Loos et al., 2000); in addition these markers (IL-6 &CRP) are well recognised as useful biomarkers for cardiovascular diseases (Danesh et al., 2000; Ridker et al., 1997).

The majority of serum-based biomarkers that have been studied in periodontal disease have focussed on the levels of serum antibodies to certain pathogens, and latterly on factors that may indicate systemic inflammation such as C-reactive protein (Chapple, 1997; D'Aiuto et al., 2004; Paraskevas et al., 2008). Therefore, in this study we were interested into two serum markers; CRP &IL-6.

1.1.6 Systemic Inflammation and Biomarkers

There has been extensive interest in recent years in the links between periodontal disease, systemic inflammation and a number of systemic conditions including CVD, diabetes, adverse pregnancy outcomes, and others such as osteoporosis, rheumatoid arthritis and chronic kidney disease. Interpreting these studies which show a positive association of periodontal disease with risk of these systemic conditions is complicated as association does not necessarily indicate a causative relation.

Cardiovascular Disease

In terms of the association of CVD with PD, there is strong and consistent epidemiological evidence from a number of systematic reviews with meta-analyses that both the prevalence and incidence of CVD is increased in subjects with periodontitis (Bahekar et al., 2007; Blaizot et al., 2009; Humphrey et al., 2008; Janket et al., 2003; Khader et al., 2004; Paraskevas et al., 2008; Sfyroeras et al., 2012). However, most of the included studies in these meta-analyses have been criticised for either lack of uniform definition of periodontitis, small sample size, or incomplete adjustment for all Framingham risk factors especially tobacco consumption. In addition many studies have shown an association between PD and biomarkers of CVD such as elevated CRP levels (D'Aiuto et al., 2004; Liu et al., 2010), endothelial dysfunction of peripheral blood vessels (Mercanoglu et al., 2004; Seinost et al., 2005; Tonetti et al., 2007) and thickness of carotid intima-media (Piconi et al., 2009). However, it was concluded in the joint workshop of

EFP/AAP on periodontitis and systemic disease that although the association between periodontitis and Atherosclerotic Cardio-Vascular Disease (ACVD) is confirmed, we still need well-designed intervention trials to investigate the effect of periodontal treatment on the prevention of ACVD (Tonetti and Dyke, 2013).

Diabetes Mellitus

There is a wealth of epidemiological and biological evidence demonstrating that diabetes mellitus (DM) is an established risk factor for periodontitis, that diabetics with poor glycaemic control are at greater risk of having progressive and severe periodontitis than those with well controlled diabetes (Chávarry et al., 2009; Emrich et al., 1991; Khader et al., 2006; Salvi et al., 2008; Taylor et al., 1996; Tsai et al., 2002), and the risk for periodontitis in diabetics is increased by up to threefold compared to non-diabetics (Mealey and Ocampo, 2007). Noticeably, most of the research has focused on type II DM due to the fact that both PD and DM historically tend to develop in subjects in their forties and fifties. However, patients with type I DM are also at risk of PD.

In addition, there is considerable discussion about whether PD may increase the risk of DM, and, following on from that if treatment of PD may improve glycaemic control in DM (Taylor, 2001).

Serum levels of the pro-inflammatory cytokine TNF-α, which is closely linked to insulin resistance (Type II diabetes), are elevated in subjects with severe periodontitis, which has suggested that treating periodontal disease in type II diabetic subjects could restore insulin sensitivity resulting in improved glycaemic control (Engebretson et al., 2007; Iwamoto et al., 2001) by reduction of both local and systemic inflammation (Mealey and Rose, 2008).

Many interventional clinical studies of diabetic subjects with severe periodontitis have shown significant improvements in their glycaemic control after standard periodontal therapy alone (Kıran et al., 2005; Rodrigues et al., 2003; Stewart et al., 2001) or with adjunctive antimicrobials (Grossi and Genco, 1998; Grossi et al., 1997; Grossi et al., 1996). However, other similar studies have not found this significant effect of periodontal therapy on glycaemic control (Engebretson et al., 2013; Janket et al., 2005; Jones et al., 2007).

Meta-analyses have concluded that treating periodontal disease in type II diabetic subjects could improve the glycaemic control (Darré et al., 2008), for at least three months (Teeuw et al., 2010). However, other reviews have criticised that these interventional studies were few and most of them were lacking the power to detect the significant effect of treating periodontitis on glycaemic control (Engebretson and Kocher, 2013; Lalla and Papapanou, 2011; Simpson et al., 2010).

In addition, in a recent systematic review of epidemiological non-interventional evidence of effects of PD on diabetes incidence, control and complication, by Borgnakke and co-workers, they concluded that the current evidence is scarce (Borgnakke et al., 2013).

Therefore, the joint workshop of EFP/AAP consensus report on periodontitis and systemic diseases reported that further research is needed to clarify these relationships and prospective, larger, controlled trials are needed in order to establish that treating periodontitis could improve the glycaemic control of subjects with diabetes (Chapple and Genco, 2013).

Obstetric Outcomes

There is also extensive research into the potential association between PD and adverse pregnancy outcomes like preterm birth, pre-eclampsia and low birth weight (Boggess et al., 2003; Chaparro et al., 2012; López, 2008; Offenbacher et al., 2006; Xiong et al., 2006). However, it is still unclear how periodontal disease can adversely affect these outcomes. Interestingly, some studies suggested that treating periodontitis during pregnancy could reduce the risk of preterm birth (Jeffcoat et al., 2003; López et al., 2002). Even more, a recent systematic review has shown that the non-surgical periodontal treatment of pregnant women (with high risk of preterm birth only) with periodontitis significantly reduce the preterm birth risk (Kim et al., 2012). On the other hand, many recent systematic reviews of large and high quality randomised clinical trials have concluded that maternal periodontal disease treatment, delivered early in pregnancy, has no effect on the adverse pregnancy outcomes (Chambrone et al., 2011; Michalowicz et al., 2013).

Therefore, the EFP/AAP joint workshop of periodontitis and systemic disease advised that further research is needed to better understanding of the

pathophysiology and risk profile that behind this link, to define the best time and type of treatment intervention, and to identify the subgroup of pregnant women who will benefit from such an intervention (Sanz and Kornman, 2013).

Based on these associations, periodontal infections might be considered a significant risk factor for some systemic diseases, and in turn control of periodontal diseases might be of great importance in the prevention and management of several systemic diseases although clear evidence of this remains inconclusive.

However there is now clear evidence that periodontal disease can cause systemic inflammation and it has been postulated that there might be a mechanistic link between these conditions. In particular the relationship between periodontal disease and circulating Reactive Protein and IL-6 has been described in a number of studies.

CRP (Circulating Reactive Protein)

C - reactive protein (CRP) is regarded as a biomarker for systemic inflammation, and is produced from hepatocytes. It is a non-specific marker of the acute phase response of innate immunity, that can be induced by many stimuli like chronic inflammation, trauma and smoking (Blake and Ridker, 2001; Blake et al., 2003). In periodontal disease, its secretion is stimulated by circulating cytokines, such as TNF-α and IL-1 (Wong, 2008). It is considered a key marker for atherosclerosis and high levels of CRP are associated with cardiovascular disease (Blake and Ridker, 2003).

Although many studies have shown that there is an association between periodontal disease and the risk of myocardial infarction (Leivadaros et al., 2005; Söder et al., 2005), this has not been shown to necessarily be a causal association. Several cross-sectional (case-control) studies have shown that CRP levels were higher in both aggressive (Havemose-Poulsen et al., 2006; Salzberg et al., 2006) and chronic periodontitis subjects than healthy controls (Fredriksson et al., 1999; Liu et al., 2010; Loos et al., 2000; Pradeep et al., 2010).

On the other hand, other studies have concluded that serum CRP concentrations were not associated with periodontal disease as there was no difference in Serum CRP levels between groups of chronic periodontitis and healthy controls (Tüter et al., 2007; Yamazaki et al., 2005).

Interestingly, many interventional longitudinal (treatment) studies using different periodontal treatment modalities have shown that serum CRP levels were reduced significantly after using periodontal treatment including the use of systemic or local sub- and antimicrobial medications (D'Aiuto et al., 2005b; D'Aiuto et al., 2004; Golub et al., 2010; Katagiri et al., 2009; Mattila et al., 2002; Renvert et al., 2009; Seinost et al., 2005). However, Offenbacher and co-workers have reported in their randomised clinical trial that serum CRP levels were not affected by root surface debridement especially when its' level was less than 3mg/L.

In a systematic review of the relationship between CRP & periodontal disease it was concluded that periodontal inflammation can induce a mild acute phase response with an increase of CRP levels, and all the included studies showed that CRP levels were elevated in periodontitis subjects compared with healthy controls. Furthermore periodontal treatment lowers CRP levels (Paraskevas et al., 2008).

In addition, a recent meta-analysis has concluded that periodontal treatment reduces CRP levels and improves endothelial function, especially in patients with CVD and diabetes (Teeuw et al., 2014).

IL-6 (Interleukin-6)

IL-6 is a major mediator of the inflammatory response which is involved in the induction of acute phase proteins and the induction of fever. It is a multifunctional pluripotent pro-inflammatory cytokine produced by many activated cell types including monocytes, B lymphocytes, endothelial cells and fibroblasts. However, it needs a stimulus to be produced and cannot be just released from intact normal cells. It is an important molecule in the regulation of immune response and host defence reaction and in the transition between acute and chronic inflammation, it is involved in many homeostasis processes including induction of immunoglobulin production, T-cell differentiation, stem cell proliferation etc. it is highly expressed in many autoimmune diseases like Rheumatoid Arthritis (RA) & psoriasis (Hirano et al., 1988), as well as in acute and chronic inflammatory disease such as sepsis and atherosclerosis (Beckman et al., 2005; Schlüter et al., 2002).

IL-6 has a potential role in driving the destructive process of periodontal disease, by acting as a pro-inflammatory cytokines alongside IL-1 & TNF-α. It is a bone

resorbing factor, and it causes a dose-dependent inhibition of bone formation *in vitro* (Hughes and Howells, 1993). Surprisingly, some studies show that IL-6 promotes soft tissue formation. In addition, IL-6 release can balance the pro-inflammatory effect of IL-1 & TNF-α, by inhibiting their production. So IL-6 may have both important destructive and protective effects (Irwin and Myrillas, 1998).

Most studies have shown that the levels of IL-6 are raised both locally in periodontally inflamed sites (Guillot et al., 1995; McGee et al., 1998) and systemically in peripheral blood of patients with periodontal disease (Buhlin et al., 2003; Loos et al., 2000). These elevated levels of IL-6 can stimulate hepatocytes to produce CRP and other Acute Phase Proteins to protect the host (Irwin and Myrillas, 1998; Loos, 2005). Moreover, Raunio and co-workers' cross-sectional study of 52 subjects with chronic periodontitis concluded that serum IL-6 levels increase with periodontal severity (Raunio et al., 2007). In contrast, a cross-sectional study on Japanese elders' cohort of 74 healthy and 84 chronic periodontitis showed there were no significant relationship between serum IL-6 levels and the severity of periodontitis (Furugen et al., 2008).

Interestingly, some studies showed that serum IL-6 levels increase immediately after subgingival instrumentation (D'Aiuto et al., 2004; Ide et al., 2004), but drop significantly after periodontal treatment and resolution of inflammation (D'Aiuto et al., 2004).

Therefore, there are number of issues emerging regarding links between local and systemic inflammation to consider in the context of diagnostic biomarkers of periodontal disease:

1. Does local inflammation correlate with systemic inflammation?
2. Are systemic inflammatory markers potentially useful biomarkers of periodontal disease?
3. Are local biomarkers useful in assessing systemic inflammation?
4. Does treatment of local inflammation affect systemic inflammation?

1.2 Summary and Aims of the Project

In summary there is considerable interest in the identification, validation and clinical application of salivary biomarkers in periodontal disease. Due to the fact that periodontal disease is a world-wide health issue and a major cause of tooth loss. Therefore, we all as periodontists are aiming to improve early diagnosis and management of periodontal disease.

The literature is rich with studies looking for biomarkers with promising periodontal diagnostic utility, and MMP-8 is the most studied one. In addition, results from previous studies in our laboratory have identified three AMPs HNP1-3, S100A8 and LL-37, using mass spectrometry techniques, as potential diagnostic biomarkers for periodontal disease (Mulli, 2012).

Consequently there is scope to extend these previous studies in the study of potential periodontal biomarkers. Firstly there is the opportunity to extend and validate our previous observations of the use of AMPs as diagnostic biomarkers, by using a standardised salivary collection method for a reference population to determine cut off points for salivary AMP concentrations, then applying them to an independent cohort with saliva samples collected using the same method in order to validate the AMP cut-off points. Furthermore combinations of biomarkers can be tested for improved performance compared with single biomarkers alone. Secondly there is the opportunity to carry out a longitudinal study of participants undergoing treatment. This would allow us to investigate the effects of treatment on the concentrations of these biomarkers. Most importantly it will also allow us to test if these biomarkers have any prognostic significance, such that their salivary concentrations may be associated with good or poor responses to initial therapy. Thirdly collection of samples during a longitudinal study will also allow the possibility of using samples to investigate the discovery of novel prognostic biomarkers.

Therefore the aims of the studies in this project are:

I. Investigate the use of a combination of the antimicrobial peptides HNP1-3, LL-37 and S100A8 and the proteolytic enzyme (MMP-8) as diagnostic biomarkers of periodontal disease.

II. Investigate the use of salivary AMPs as prognostic biomarkers in longitudinal studies of nonsurgical periodontal treatment;

III. Investigate the association between putative salivary biomarkers and systemic markers.

CHAPTER 2- VERIFICATION & VALIDATION OF SALIVARY BIOMARKERS AS DIAGNOSTIC PERIODONTAL BIOMARKERS

2. Verification and validation of salivary biomarkers as diagnostic periodontal biomarkers

2.1 Overview

In a previous study in our lab Dr.T Mulli identified three AMPs (HNP1-3, S100A8 and LL-37) as potential diagnostic biomarkers, using mass-spectrometry techniques. Unfortunately, he was unable to validate his results in an independent cohort of subjects, due to the different saliva collection methods that were used. In the original cohort of Dr. Mulli a salivette technique was used to collect the saliva, while in the independent cohort a spitting method was used. He investigated the difference between the two methods and discovered that the salivary AMPs concentrations collected by salivette technique were 10 times less than the ones collected by spitting methods (Mulli, 2012).

Therefore, in order to validate these recently identified AMPs as potential biomarkers for periodontal disease, first we have to determine a cut-off point for every biomarker. This also would allow us to confirm these original findings in a new cohort of samples collected by the spitting method selected on the basis of their periodontal diagnosis. Then we could compare the results of AMPs with salivary MMP-8 concentrations, which appear to be the best published marker for periodontal disease to date. In addition, we can investigate the value of combining biomarkers. Once all these steps were done we can validate our result in an independent cohort.

To validate means to test the external validity of results obtained in one experiment. Specifically, this means to test if the results obtained are also applicable in independent samples. Therefore, validation studies are crucial as it is the only way to have a novel, feasible and accurate technology in order to attain definitive point-of-care assessment of individuals` periodontal health status (Li et al., 2005).

In general, there is a need for better biomarkers in order to enhance diagnosis/prognosis and monitor the treatment response in many diseases.

Although proteomic techniques give a hope for discovery of novel biomarkers, the results are not satisfactory so far. This is mainly due to the lack of proper validation methods (Rifai et al., 2006).

The validation process is the last, though not the least, step in the development of novel protein biomarker candidates (Rifai et al., 2006) Figure 2-1. The steps briefly are:

1. Discovery: which means the identification of candidate biomarkers that is differentially expressed between health and disease.
2. Qualification: This confirms the differential abundance of the discovered candidate in human sample.
3. Verification: in which the sensitivity of biomarker candidate is confirmed, and specificity commences to be tested.
4. Validation and clinical assay development: where sensitivity and specificity are established, and assay is optimized.

Figure 2-1: Flow chart illustrating the steps for the development of novel protein biomarker candidate (Acknowledgment: (Rifai et al., 2006)**).**

The validation process aims to investigate the validity of the identified biomarkers against a heterogeneous mass of patients, to ensure its' diagnostic utility. Consequently, establishing a test threshold for disease detection by providing test sensitivity and specificity. Therefore, in this study we used Receiver Operating Characteristic (ROC) curve in order to determine the best cut-off point for a given biomarker values .i.e. where to draw the line between health and disease for example.

The ROC curve is an informative and fundamental tool for diagnostic test evaluation. In a ROC curve the true positive rate or values (TPR or sensitivity) is plotted on the (y-axis) against false positive rate or values (FPR or 1- specificity)

on the (x-axis) for different cut-off points of a parameter Figure 2-2. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. Maximising sensitivity corresponds to some large (y) value on the ROC curve, while maximising specificity corresponds to a small (x) value on the ROC curve. That is why the best cut-off point choice is the value that correspond to a point nearest to the upper left corner on the ROC curve graph, as shown on Figure 2-2; the perfect classification point (0,1). The area under the ROC curve (AUC) is a measure of how good a parameter can differentiate between two diagnostic groups (healthy/diseased) (Zweig and Campbell, 1993).

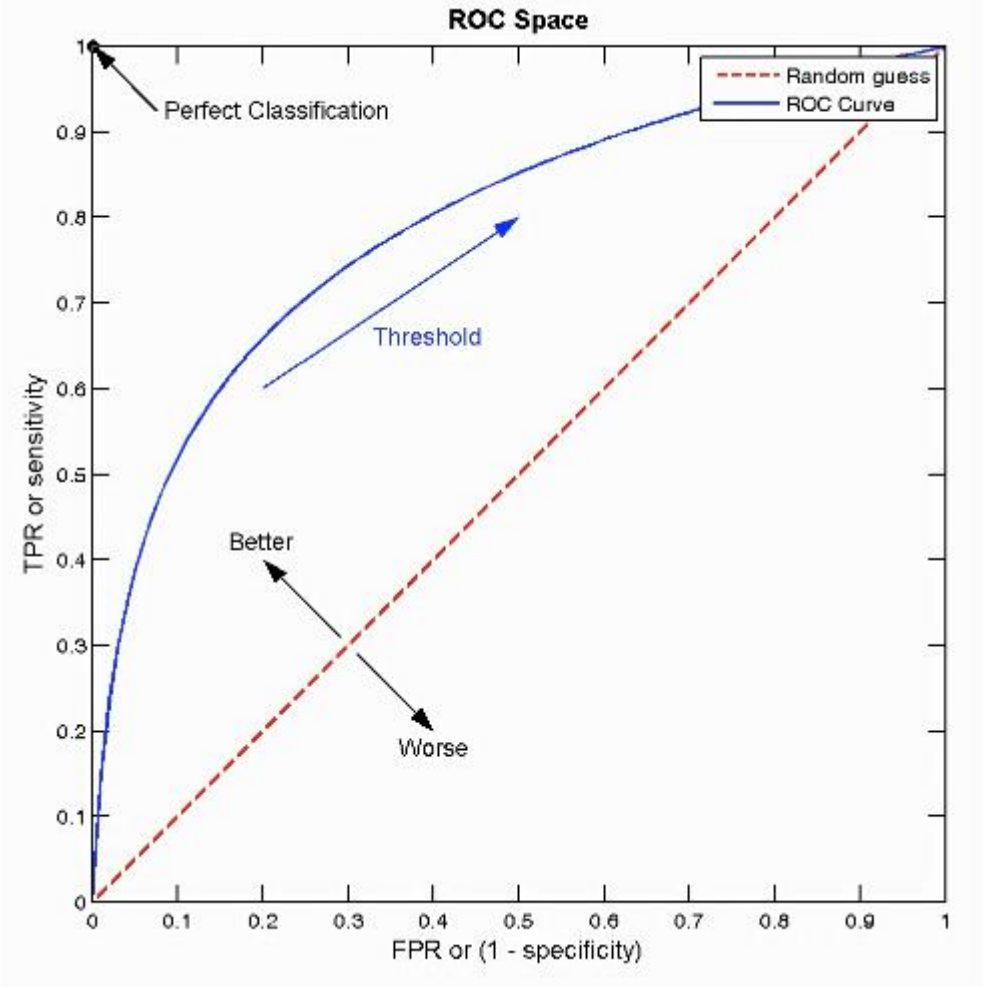

Figure 2-2: ROC curve graph showing the dimensions that determine the classification of a test as either perfect, better or hopeless. (Acknowledgement: (Online Wikipedia, ROC curve)).

***FPR= False Positive Rate, *TPR= True Positive Rate**

There is a wealth of research in the literature suggesting MMP-8 as a potential biomarker for periodontal disease for health and disease (Adina Bianca Boşca, 2012; Sorsa et al., 2011; Sorsa et al., 2004). Therefore it is also of considerable relevance to compare our own putative biomarkers with MMP-8.

In addition, it has been suggested that it may be useful or necessary to combine the findings of different biomarkers in order to improve the utility of a diagnostic test.

2.2 Aims & Objectives

Therefore the aims of this cross sectional study were:

1. To identify suitable cut-off points for diagnostic testing for periodontal disease of the salivary AMPs: HNP1-3, S100A8 & LL-37.
2. To compare the diagnostic utility of salivary MMP-8 with the salivary AMPs tested.
3. To investigate the periodontal diagnostic utility of combined salivary biomarkers (MMP-8 + HNP1-3 + S100A8, MMP-8+HNP1-3, MMP-8+S100A8, and HNP1-3+S100A8) compared to individual markers.
4. To validate these biomarkers as putative diagnostic markers for periodontal disease in an independent cohort of subjects.

2.3 Materials and Methods for the Original Cohort

2.3.1 Ethical Approval

The protocol for this study was reviewed and approved by the Outer North East London Research Ethics Committee on 27th May 2010, reference number: (10/H0701/72). All the participants signed an informed written consent form.

2.3.2 Inclusion Criteria

The target patients (males and females of any ethnicity), who had been included in this study, were between the ages 18 to 65 years and had periodontal disease (G, ChP or AgP). Smokers and diabetics were included.

Case Definitions

Moderate and severe Chronic Periodontitis were defined as below from the CDC /AAP criteria (Page and Eke, 2007) as described in Chapter 1(1.1.2):

a) Moderate Periodontitis:

    a. Patients with two or more interproximal sites with clinical attachment loss of ≥ 4mm occurring at two or more different teeth OR two or more interproximal sites with a probing depth of ≥ 5mm, not on the same tooth.

b) Severe Periodontitis:

    a. Patients with two or more interproximal sites with clinical attachment loss of ≥ 6mm not on the same tooth, and the presence of one or more interproximal sites with a probing depth of ≥ 5mm.

Aggressive periodontitis cases were defined according to (Demmer and Papapanou, 2010) suggestion which they base on that this severity of destruction would not be commensurate with the amount of plaque present irrespective of plaque control:

a) In patients ≤25 years of age, the presence of two or more interproximal, nonadjacent sites with attachment loss of ≥4 mm occurring at a minimum of two different teeth and accompanied by bleeding on probing, will signify aggressive periodontitis.

b) In individuals between 26 and 35 years of age, a diagnosis of aggressive periodontitis will require the presence of two or more interproximal, nonadjacent sites with attachment loss of ≥6 mm occurring at a minimum of two different teeth and accompanied by bleeding on probing.

In terms of disease distribution, both localised (≤30% of sites involved) and generalised (>30% of sites involved) types were included as long as they are following the above mentioned case definitions.

While for the control group (gingivitis /mild periodontitis case) patients with less than two sites with clinical attachment loss of ≥ 4mm and bleeding on probing.

2.3.3 Exclusion Criteria

Patients were excluded if they had previous periodontal treatment (including antimicrobials) in the last 6 months, were taking any relevant medication or had any systemic disease known to affect the periodontal tissues.

2.3.4 Sample Acquisition

Based on our previous pilot data we calculated that a sample size of 35 in each group would give 80% power at $P < 0.05$, to detect a 60% mean change in HNP concentration between control and diseased samples assuming a mean of 3µg/ml in control samples and standard deviation of 3.

A total of 133 unstimulated saliva samples were used in this study out of which 84 samples were collected as disease groups, 46 with chronic periodontitis and 38 with aggressive periodontitis from new patients attending the periodontal consultant`s clinic at Guy`s Dental Hospital. A total of 49 unstimulated saliva samples with gingivitis/mild periodontitis were used as the control group ; 19 samples were collected from new patients attending the periodontal consultant's clinics at Guy's Dental Hospital and 30 samples from non-diabetic patients with gingivitis (control group) were obtained from the cohort of samples given to us by Dr.Penny Hodge and colleagues from University of Glasgow (Hodge et al., 2012) (refer to the next section for more details regarding material and methodology of this group). Unfortunately, AMP data were not available for all of the control group samples as some samples were too depleted to complete all analyses.

The whole saliva was collected using the spitting method (by asking the participant to spit in a laboratory universal tube for 5 min). Samples were processed (centrifuged at 3000 rpm for 15min at 4 ^{o}C and aliquots of approximately 300µl)

then stored immediately in minus 80°C freezer until time for analysis. Some of the samples were collected by Dr. W-C Cheng and Miss J Tebbutt (third year undergraduate dental student at King's). The saliva sample acquisition method was the same for all groups.

2.3.5 ELISAs

ELISAs for MMP-8, HNP1-3, LL-37 and S100A8 were carried out on all saliva samples. To quantify MMP-8, the human total MMP-8 DuoSet kit (R & D Systems, inc. USA) was used according to the manufacturer's instructions. HNP1-3 and LL-37 proteins commercial kits for HNP1-3 and LL-37 (Hycult biotechnology b.v The Netherlands) were used, while Circulex S100A8 (Human) ELISA kit (CYclex Co., Ltd.Terasawaoka, Japan) commercial kit was used to quantify S100A8 protein according to manufacturer's instructions (See Appendix 5.1.1 for detailed protocols).

In brief, saliva samples and ELISA reagents were thawed at room temperature. The samples for saliva were diluted at an optimized dilution factor of (X 300) for MMP-8 (Serial Dilution D1= 1:10, D2=1:30), (X 5000) for HNP1-3 (Serial Dilution D1=1:250, D2=1:20), (x 5) for LL-37 and (x 100) for S100A8 (with second reading of the plates at 405nm for high concentration samples). Both standards and samples were run in duplicates. Colorimetric reading was done using Dynex Revelation® 4.24 at 450nm. Standard curves were plotted using Microsoft Windows® Excel 2010 spreadsheet software.

2.3.6 Data Analysis

Statistical analysis was carried out using Prism® 6 (v 6.04) for Windows MS (GraphPad Software Inc., California, USA). One-way analysis of variance (ANOVA) was used to analyse the difference in expression of more than two analytes in different periodontal disease. While in case where only two analytes were tested, two-tailed unpaired t-test was performed. Statistical significance was set at $p \leq 0.05$.

Receiver Operating Characteristic Curves (ROC curve) were constructed for those analytes that showed a significant difference in expression, and cut-off points determined for optimum utility to discriminate between gingivitis and periodontitis. In general cut-off points were chosen particularly to maximise specificity, even at the expense of some sensitivity. In addition, biomarkers were combined to give a single cut off point, using two different methods:

1. Normalised Data Method (NDM):

In order to combine the biomarkers objectively each value for all biomarkers was normalised by dividing the actual concentration by the optimal (predetermined) cut off value such that an original biomarker cut-off point concentration was normalised to a score of 1 Figure 2-3. The new normalized values for every sample were added together to form a new combined value. These new set of data were used to construct a new ROC curve, which produced a combined sensitivity and specificity at any combined cut-off point.

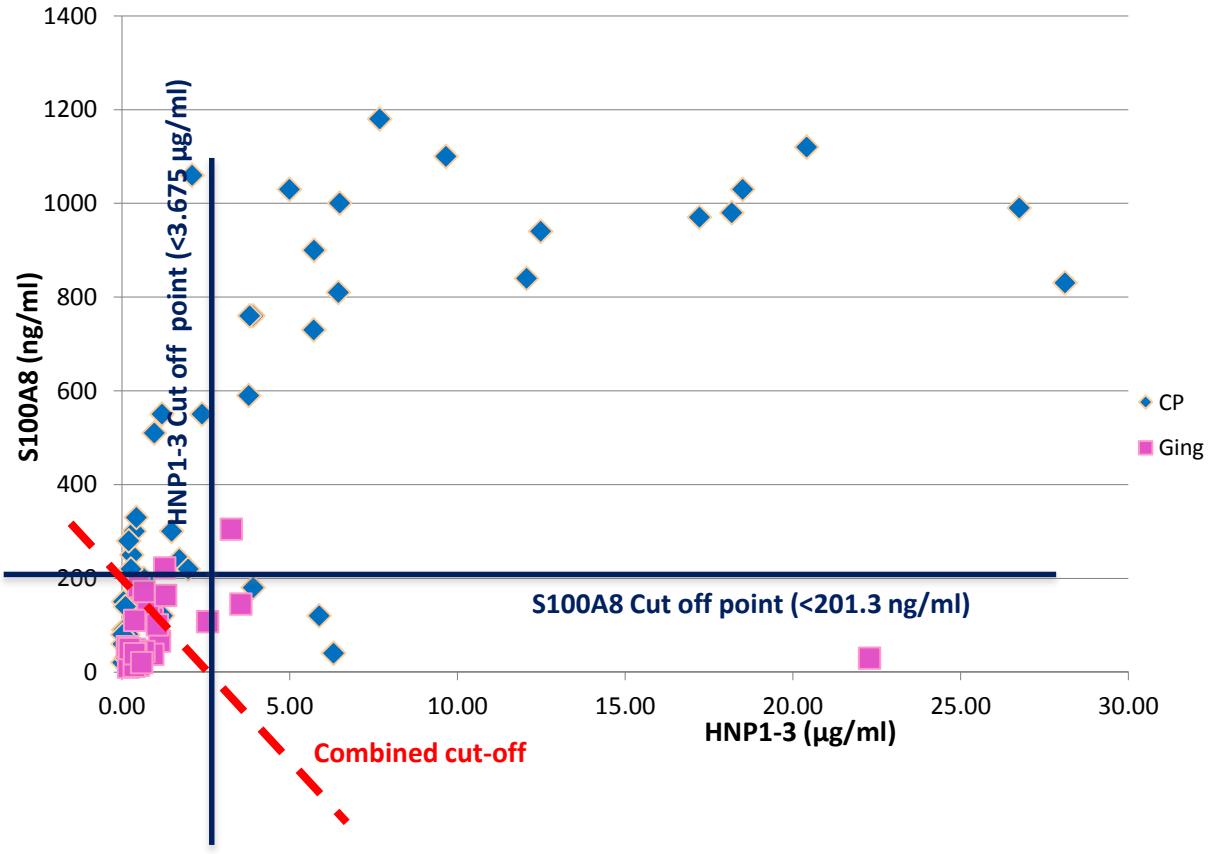

Figure 2-3: Diagram showing the principles of the Normalised Data Method for combining biomarkers. After normalising data for each biomarker so that the cut-off point of each biomarker = 1, the results for each biomarker are added to give a combined cut off point, in this case the value 1. In the example here the actual values of 2 biomarkers are combined (HNP1-3 (x) +S100A8 (y) = combined value); where 3 biomarkers are to be combined, they are all added together (x+y+z = combined value).

2. Any Positive Value Test (APVT):

This involves assigning each analyte sample result as positive or negative based on the pre-chosen cut-offs; so if the AMPs concentration of the sample was equal or below the cut-off point then the sample would be assigned as negative otherwise it would be positive. Then in order to combine both AMPs we consider the combined marker for each sample to be positive if any of the tested biomarkers were positive. The combined marker will be negative only when all biomarkers are negative. By Using GraphPad Prism 6 software to analyse the contingency table using Fisher`s exact test, the sensitivity and specificity of the combined biomarker can be achieved.

These cut-off points of the combined data were subsequently applied to salivary biomarkers' levels in an independent cohort originally collected to investigate effects of diabetes on periodontitis.

2.4 Material & Methods for the Independent Cohort

For the control and independent groups, the saliva samples were generously given by Dr. Penny Hodge and colleagues of University of Glasgow. These samples were collected as part of a study of the effect of type I diabetes on risk of periodontitis, so consequently were chosen for their diabetic status (no diabetes, Type I DM with good glycaemic control, Type I DM with poor glycaemic control) and then only subsequently received a periodontal assessment.

2.4.1 Ethical Approval

The protocol for that study was approved by the local research ethics committee,(Glasgow Royal Infirmary Research Ethics Committee, NHS Greater Glasgow and Clyde; Reference number: 05/S0705/70).

2.4.2 Inclusion Criteria

As mentioned earlier, the recruitment for this group was based on patients` diabetic and not periodontal status. Subjects who had been diagnosed with type I diabetes for a minimum of 5 years, were aged between 20 and 55 years and had been non-smokers for a minimum of 5 years before being enrolled in the study. blood samples were taken from the control participants to confirm they were definitely controls and not in pre-diabetic state (Glycosylated haemoglobin (HbA1c) < 5.9%). All participants had to have not less than 20 natural teeth (Hodge et al., 2012).

Cases were classified as healthy, gingivitis/mild periodontitis (control group), moderate periodontitis or severe periodontitis (disease group) based on the number of sites with predetermined threshold of clinical attachment loss (CAL) Table 2-1.

Diagnosis	Criteria
Gingivitis/ Mild periodontitis	< 2 sites with CAL***** ≥ 4mm & BoP******
Moderate periodontitis	≥ 2 sites with CAL ≥ 4mm but < 6mm
Severe periodontitis	≥ 2 sites with CAL ≥ 6mm

*CAL = Clinical Attachment Loss, **BoP = Bleeding on Probing

Table 2-1: summary of the case definition criteria.

2.4.3 Exclusion Criteria

Any participant with any of the following criteria was excluded from the study: pregnant, immunosuppressant, on medications with side effect on oral cavity, on antibiotics or anti-inflammatory drugs within the previous 6 weeks and who had less than 20 teeth and smoking for the last 5 years.

2.4.4 Sample Acquisition

A total of 193 saliva samples with well or poorly controlled diabetes and non-diabetics, of this independent cohort, were used in this study, which overall included 131 subjects with gingivitis/mild periodontitis, 48 subjects with moderate periodontitis and only 14 subjects with severe periodontitis. The saliva sample acquisition method was the same as for the original cohort.

2.4.5 ELISAs

The ELISA tests for the salivary biomarkers of the independent cohort were carried out by Dr. T.Mulli and some by Dr. N.Imam (under my supervision) and Dr. O.Patel, using the same commercial kits and following the manufacturer's protocols that were used for the original cohort saliva samples.

2.5 Results

2.5.1 Periodontal diagnostic utility of Individual salivary antimicrobial peptides

HNP1-3

There were a total of 103 saliva samples available for HNP1-3 analysis using ELISA; 48 with gingivitis, 41 with chronic periodontitis and 14 with aggressive periodontitis.

The mean concentration of HNP1-3 in gingivitis was 1.65 µg/ml (SD 3.40 µg/ml); chronic periodontitis was 9.05 µg/ml (SD 9.30 µg/ml); and aggressive periodontitis 10.17 µg/ml (SD 9.05 µg/ml).

The three diagnostic variables were statistically analysed using one-way Analysis Of Variance (ANOVA) test which revealed significant differences in the HNP1-3 levels of chronic periodontitis and aggressive periodontitis compared to gingivitis ($P<0.0001$). There was no significant difference in HNP1-3 levels between chronic and aggressive periodontitis ($p=0.5$) Figure 2-4.

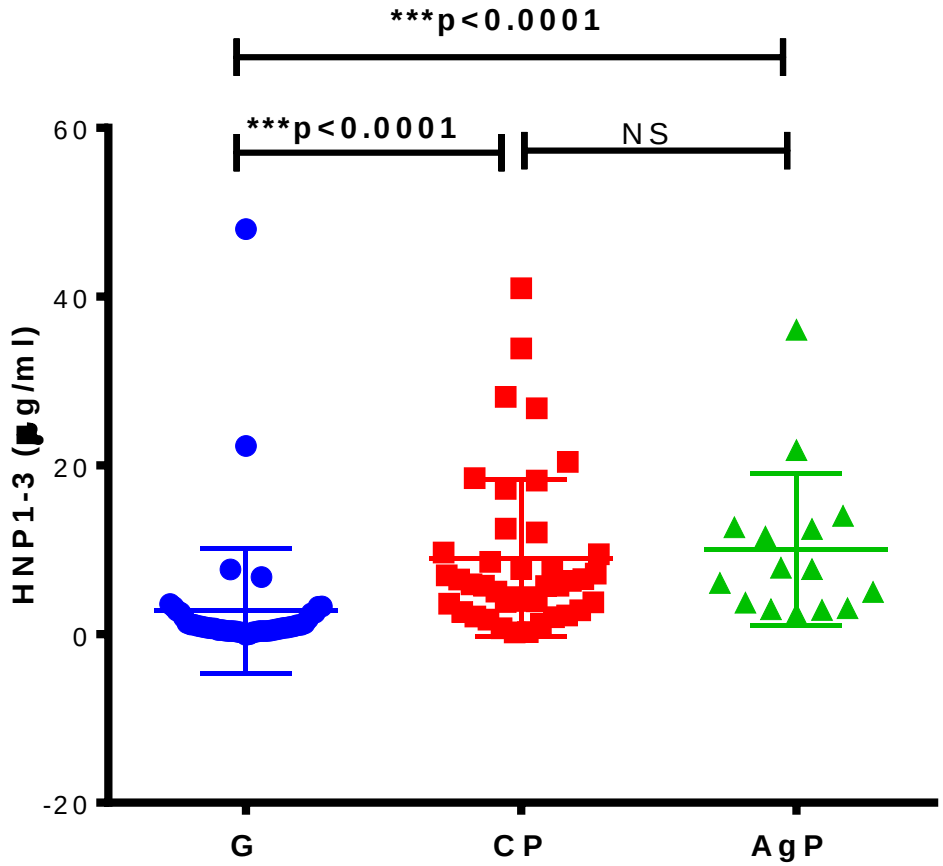

Figure 2-4: Vertical scatter graph of salivary HNP1-3 concentrations measured using ELISA, showing greater levels in both chronic periodontitis (CP) and aggressive periodontitis (AgP) than in gingivitis (G) by ANOVA.

ROC curves were constructed for the data (Gingivitis vs Chronic Periodontitis, and Gingivitis vs Aggressive Periodontitis) and showed that HNP1-3 can differentiate between gingivitis and chronic periodontitis with specificity of 91.84% and sensitivity of 73.81% at a cut-off salivary HNP1-3 concentration of > 3.675 µg/ml ($p<0.0001$) Figure 2-5. In addition, HNP1-3 was able to discriminate between gingivitis and aggressive periodontitis at cut-off salivary HNP1-3 concentration of > 2.815 µg/ml, with 83.67% specificity and 93.33% sensitivity ($p<0.0001$) Figure 2-6.

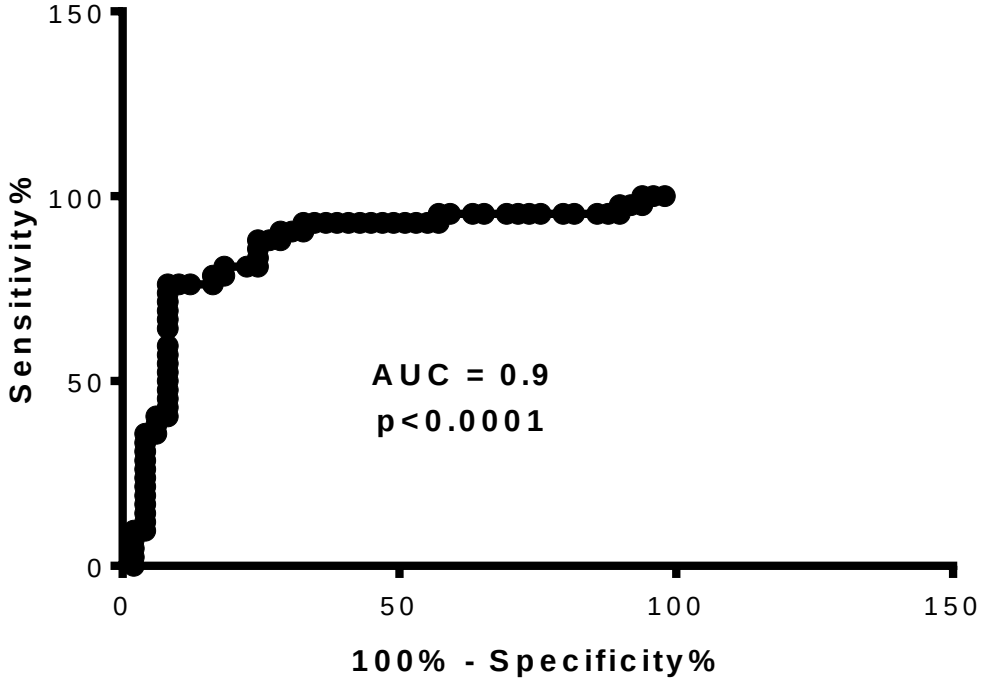

Figure 2-5: ROC curve construct for salivary HNP1-3 that distinguish between gingivitis and chronic periodontitis.

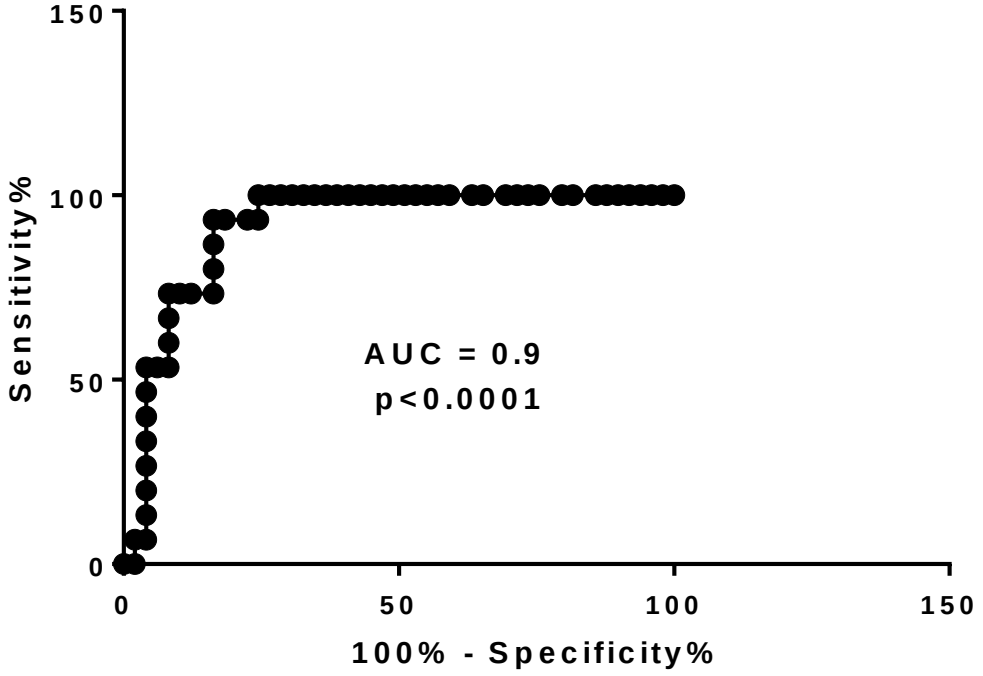

Figure 2-6: ROC curve construct for salivary HNP1-3 to distinguish between gingivitis and aggressive periodontitis.

LL-37

There were a total of 113 saliva samples available for LL-37 ELISA analysis; 37 cases with gingivitis, 39 with chronic periodontitis and 37 with aggressive periodontitis.

The mean concentration of LL-37 for gingivitis was 48.83 ng/ml (SD 54.75 ng/ml), chronic periodontitis was 40.21 ng/ml (SD 60.22 ng/ml) and aggressive periodontitis was 32.74 ng/ml (SD 27.22 ng/ml). The three diagnostic variables were statistically analysed using one way Analysis Of Variance (ANOVA) test which revealed no significant difference in the LL-37 levels of the three groups (p=0.1).i.e chronic periodontitis and aggressive periodontitis compared to gingivitis (p=0.5 & p=0.1 respectively), or between chronic and aggressive periodontitis (p=0.5) Figure 2-7.

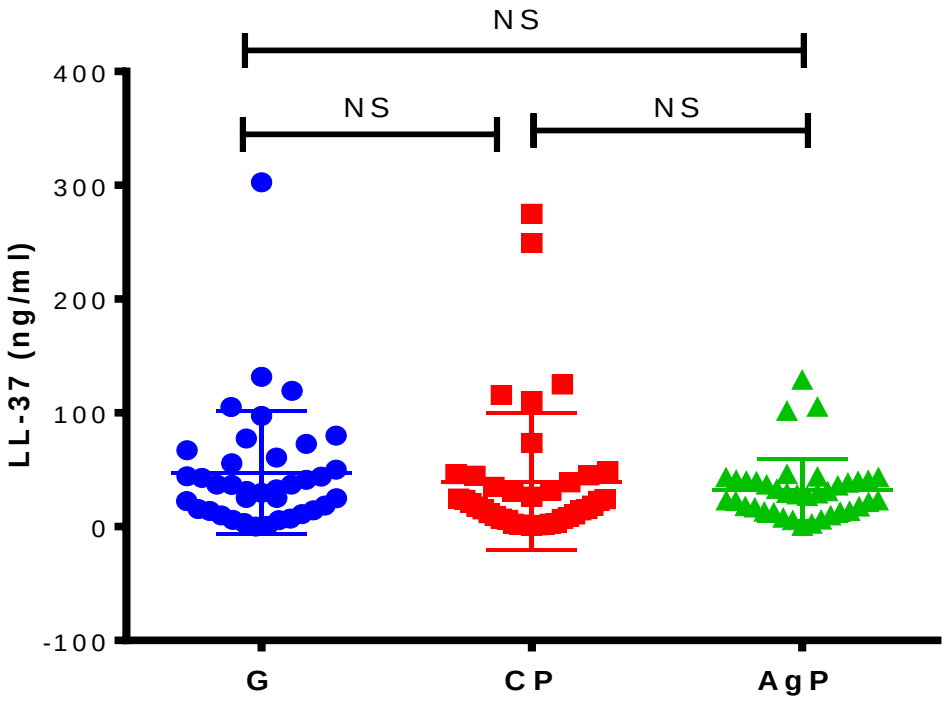

Figure 2-7: Vertical Scatter graph of salivary LL-37 showing no statistical significant differential expression in Gingivitis (G) vs. Chroinc Periodontitis (CP), (G) vs. Aggressive periodontitis (AgP) and (CP) vs. (AgP) by ANOVA.

S100A8

There were a total of 52 saliva samples available for analysis by S100A8 ELISA; 27 cases with gingivitis and 25 cases with chronic periodontitis. Increased concentrations of salivary S100A8 were seen with increased severity of periodontal disease. There was a significant difference between gingivitis/mild periodontitis (88.05 ng/ml ± 181.50 ng/ml; Mean ± SD) and chronic periodontitis (696.60 ng/ml ± 54.61402.32 ng/ml; Mean ± SD) (unpaired t-test; two-tailed p value ($p<0.0001$))

Figure 2-8.

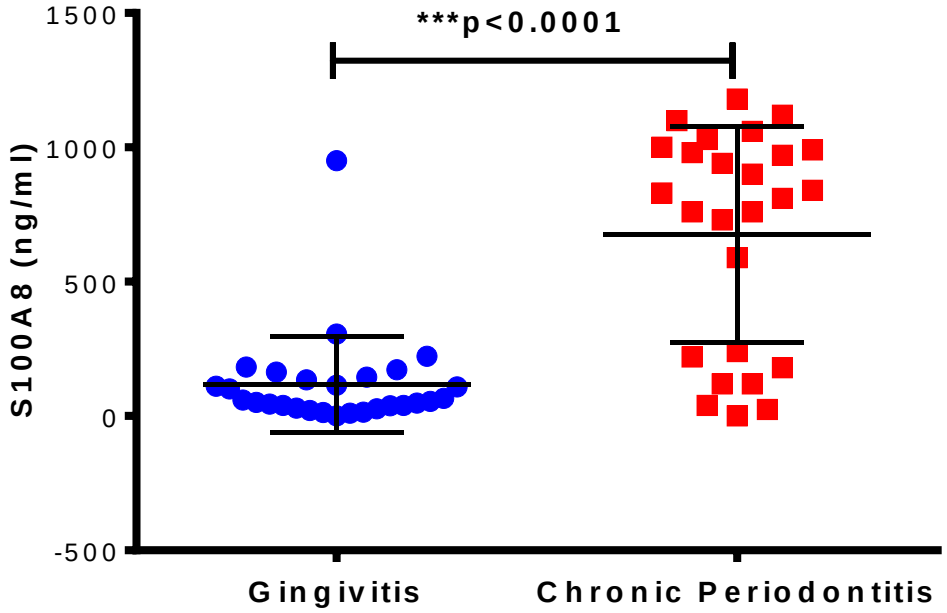

Figure 2-8: Vertical Scatter graph showing statistically significant differential expression of salivary S100A8 in Gingivitis vs. Chronic Periodontitis, by unpaired t-test (P value is two-tailed).

ROC curves were constructed and showed that S100A8 was able to differentiate between gingivitis and chronic periodontitis cases with 89.29 % specificity and 76.92 % sensitivity at a cut-off salivary S100A8 concentration of > 201.3 ng/ml (AUC=0.9, p<0.0001) Figure 2-9.

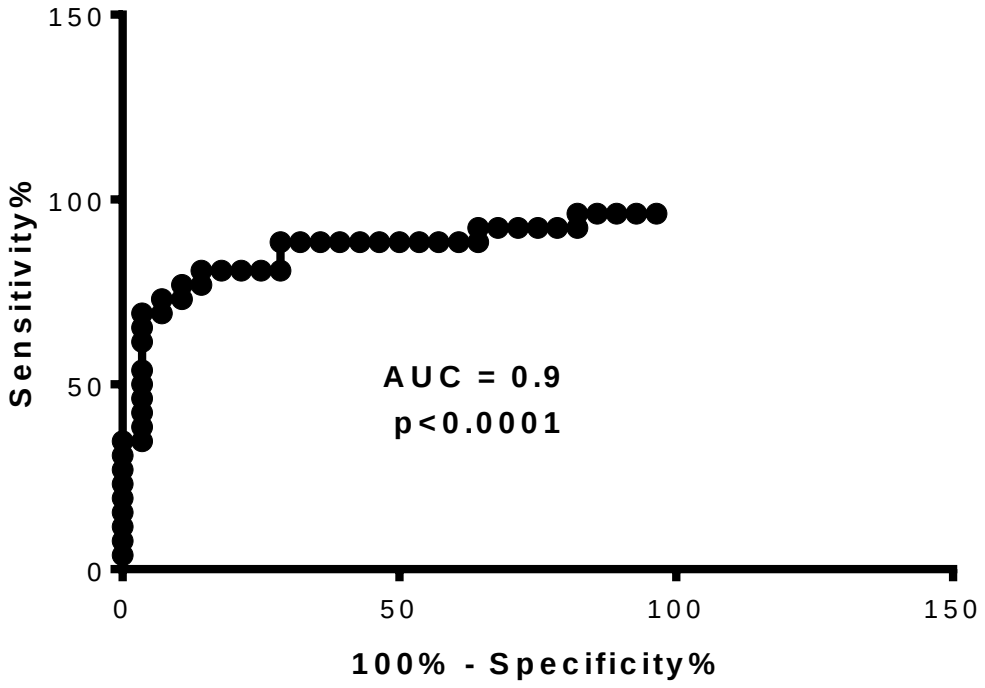

Figure 2-9: ROC curve construct for salivary S100A8 with significant ability to distinguish between gingivitis and chronic periodontitis.

MMP-8

There were a total of 128 saliva samples available for MMP-8 analysis using ELISA; 47 with gingivitis, 45 with chronic periodontitis and 36 with aggressive periodontitis.

The mean concentration of MMP-8 in gingivitis was 37.73 ng/ml (SD 75.79 ng/ml); chronic periodontitis was 358.69 ng/ml (SD 344.27 ng/ml); and aggressive periodontitis 342.49 ng/ml (SD 380.95 ng/ml).

The three diagnostic variables were statistically analysed using one-way Analysis Of Variance (ANOVA) with Bonferroni's post-test which revealed significant differences in the MMP-8 levels of the three groups (p<0.0001).i.e. chronic periodontitis and aggressive periodontitis compared to gingivitis (P<0.0001). However, there was no significant difference in MMP-8 levels between chronic and aggressive periodontitis (p=0.9)

Figure **2-10**.

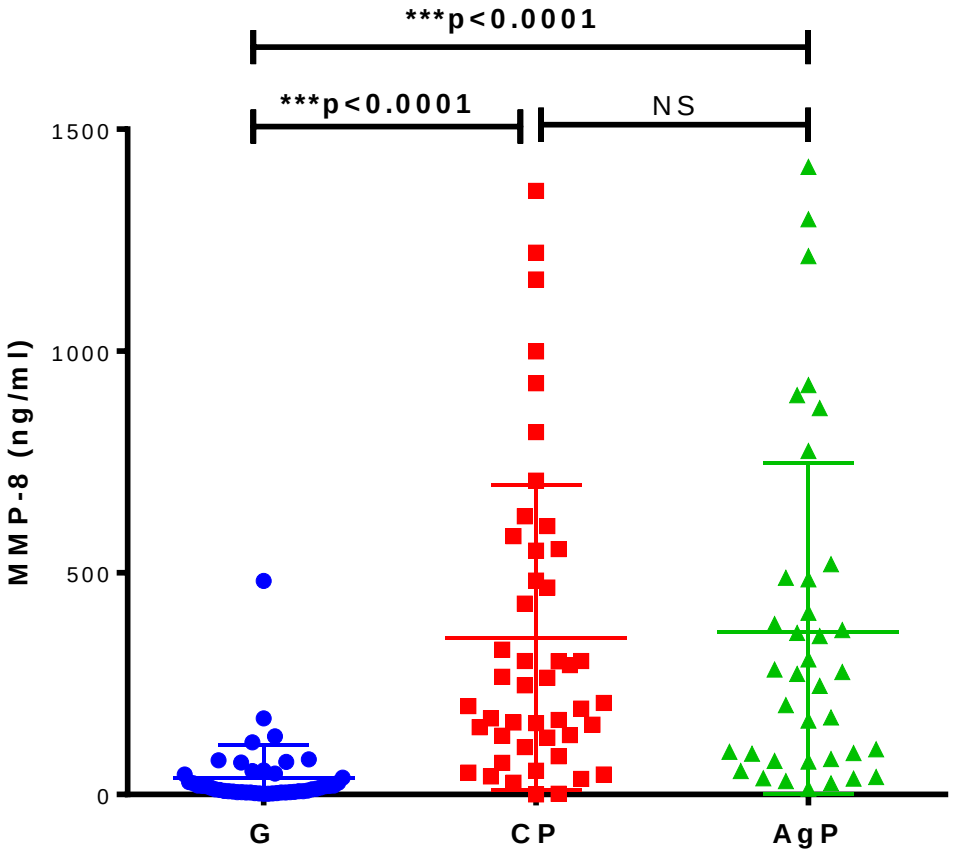

Figure 2-10: Vertical scatter graph of salivary MMP-8 concentrations measured using ELISA, showing greater levels in both chronic periodontitis (CP) and aggressive periodontitis (AgP) than in gingivitis (G) by ANOVA.

ROC curves were constructed for the data (Gingivitis vs Chronic Periodontitis, and Gingivitis vs Aggressive Periodontitis) and showed that MMP-8 can differentiate between gingivitis and chronic periodontitis with specificity of 91.73% and sensitivity of 80.43% at a cut-off salivary MMP-8 concentration of > 82.73 ng/ml (p<0.0001) Figure 2-11. In addition, MMP-8 was able to discriminate between

gingivitis and aggressive periodontitis at cut-off salivary MMP-8 concentration of > 79.73 ng/ml, with 91.67% specificity and 75.68% sensitivity (p<0.0001) Figure 2-12.

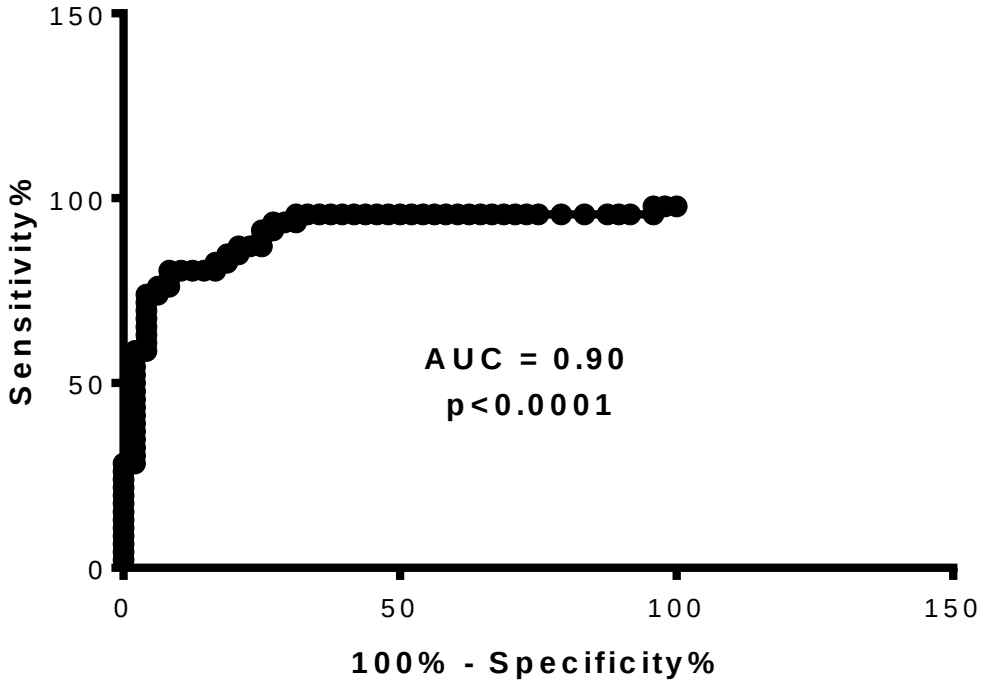

Figure 2-11: ROC curve construct for salivary MMP-8 to distinguish between gingivitis and chronic periodontitis.

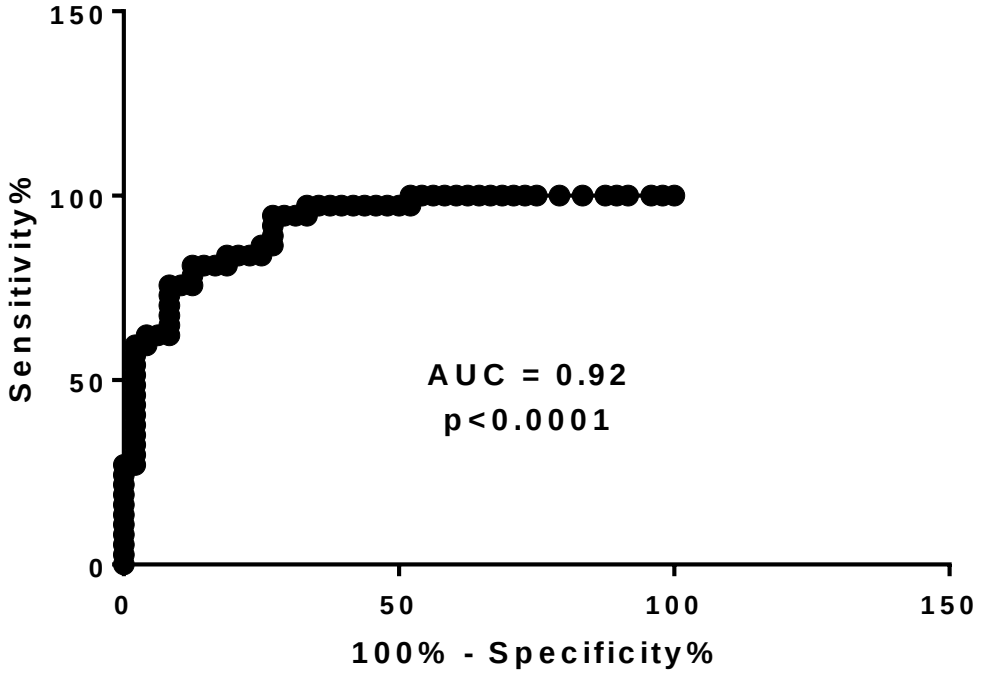

Figure 2-12: ROC curve construct for salivary MMP-8 to distinguish between gingivitis and aggressive periodontitis.

A summary of the results of the salivary biomarkers are shown in Table 2-2:

Salivary Biomarker	Cut-off	Specificity (%)	Sensitivity (%)	AUC (%)	P value
HNP1-3	>3.675 µg/ml	91.84	73.81	90	**<0.0001***
S100A8	>201.3 ng/ml	89.29	76.92	90	**<0.0001***
MMP-8	>82.73 ng/ml	91.67	80.43	90	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-2: Summary of the cut-off point concentrations of salivary HNP1-3, S100A8 and MMP-8 that significantly differentiate between Gingivitis and Chronic Periodontitis.

In addition, a summary of the number of analysed samples for each biomarker, which varied between tests because of either an inadequate remaining volume of some samples to run ELISAs for all biomarkers, or some missing AMP control data, is shown in Table 2-3:

Salivary Biomarker	Controls	Chronic Periodontitis	Total
HNP1-3	48	41	89
LL-37	37	39	76
S100A8	27	25	52
MMP-8	47	45	92

Table 2-3: summary of the number of saliva samples that were available for ELISAs analysis for every salivary biomarker.

The (AgP) cases were not considered in further analyses firstly as they did not show any significant difference from CPs, and furthermore there were no AgP cases in the independent validation cohort.

2.5.2 Testing the diagnostic utility of the composite biomarker

After investigating the diagnostic utility of the salivary biomarkers MMP-8, HNP1-3 and S100A8 individually, to the results of combining two or more of these biomarkers in order to create a composite biomarker was tested. Each single biomarker had a certain cut-off point that had been chosen with its' related sensitivity and specificity. As many recent studies have advised that the use of multiple periodontal biomarkers' combined specificity and sensitivity data might result in more predictive ROC curve than the use of single biomarkers (Gursoy et al., 2011; Kinney et al., 2011; Ramseier et al., 2009).

(MMP-8+HNP1-3+S100A8) Composite Biomarker

A total of 50 cases were available; 25 gingivitis and 25 chronic periodontitis cases with complete AMPs data for MMP-8, HNP1-3 & S100A8. The cut off points identified earlier in this chapter 2.5.1 were applied to this data (> 82.73 ng/ml for MMP-8, > 3.675 µg/ml for HNP1-3 and > 201.3 ng/ml for S100A8).

Using the first method (Normalised Data Method, NDM) for combining biomarkers (described previously) each data point was normalized to its' pre-chosen cut-off point then all 3 biomarkers for the same sample were added together. The combined sets of data were used to construct a new ROC curve that produced statistically significant ability to discriminate gingivitis from chronic periodontitis with combined specificity of 96% and sensitivity of 92% at a combined cut-off point of > 2.512 with AUC 97% Table 2-4, Figure 2-13.

Salivary Biomarker	Cut-off	Specificity (%)	Sensitivity (%)	AUC (%)	P value
Composite Marker (MMP-8+HNP1-3+S100A8)	>2.512	96	92	97	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-4: showing the different parameters of the composite marker (MMP-8+HNP1-3+S100A8) cut-off point concentration that significantly differentiate between Gingivitis and Chronic Periodontitis.

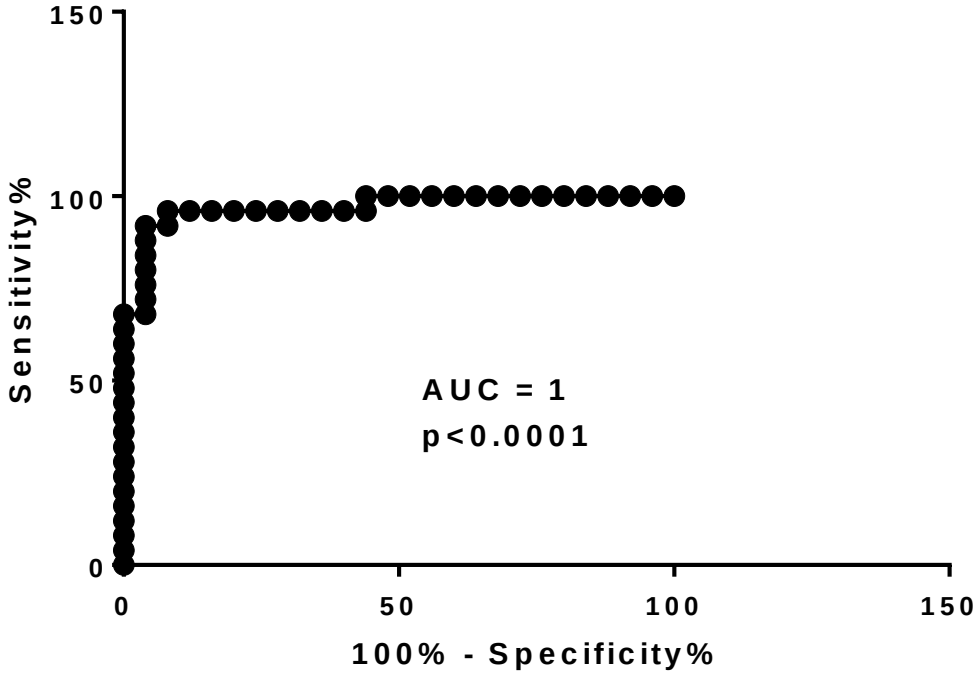

Figure 2-13: ROC curve for the combined normalised values for MMP-8, HNP1-3 and S100A8 using cut-offs 82.73 ng/ml for MMP-8, 3.675 µg/ml for HNP1-3 and 201.3 ng/ml for S100A8.

Unpaired t-test analysis of the combined new sets of data (normalised) showed that the composite biomarker was able to significantly differentiate gingivitis from chronic periodontitis ($p<0.0001$)

Figure 2-14.

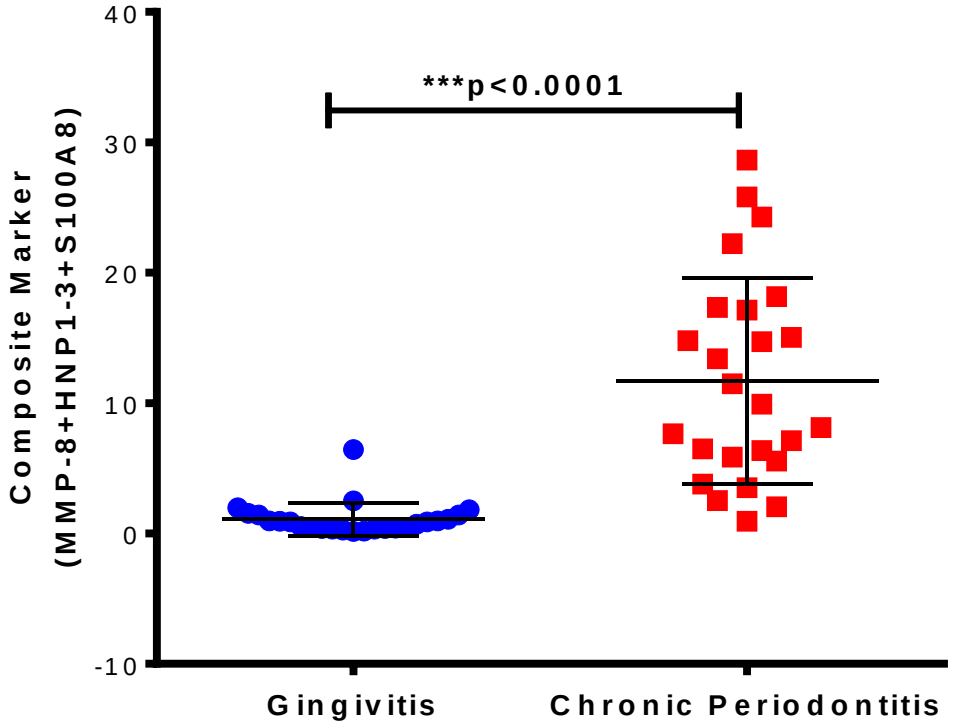

Figure 2-14: Vertical Scatter plot of the summed normalized values of MMP-8, HNP1-3 and S100A8 showing statistically significant higher mean values in chronic periodontitis, using unpaired t-test (two-tailed p value)(p<0.0001).

Using the second method (Any Positive Value Test, APVT) of combining biomarkers (described before) every sample was assigned as negative (when sample concentration ≤ cut-off point) or positive (when sample concentration > cut-off point) according to the pre-chosen cut-off points (82.73 ng/ml for MMP-8, 3.675 µg/ml for HNP1-3 and 201.3 ng/ml for S100A8) and then added together. The combined sample was considered positive if at least one of the markers was positive and negative only when all the markers were negative

Table 2-5. Fisher`s exact test contingency table analysis, gave a high specificity of 88.89% and sensitivity of 95.65% Table 2-6.

	Negative	Positive
Gingivitis	22	3
Chronic Periodontitis	1	24

Table 2-5: A contingency table of the combined (MMP-8, HNP1-3 & S100A8) for cases with Gingivitis and Chronic Periodontitis (based on predetermined cut-off points).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(MMP-8+HNP1-3+S100A8)	88.89	95.65	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-6: showing the specificity and sensitivity of the combined biomarker (MMP-8, HNP1-3 & S100A8) using Fisher`s exact test.

By using this combining biomarker method we were able to detect three CP cases more than either biomarker individually. In more detail, the combined marker detected three CP cases more than MMP-8 or HNP1-3 alone, and four CP cases than S100A8 alone.

(HNP1-3 +S100A8) Composite Biomarker

A total of 50 cases were available; 25 gingivitis and 25 chronic periodontitis cases with complete AMPs data for HNP1-3 & S100A8. As shown earlier in this chapter 2.5.1 that the thresholds were > 3.675 µg/ml for HNP1-3 and > 201.3 ng/ml for S100A8.

Using the first method (NDM) for combining biomarkers (described before), an ROC curve was conducted for the new set of normalised summed data. The composite biomarker (HNP1-3+S100A8) was able to significantly differentiate between gingivitis and chronic periodontitis with 92% specificity and 84% sensitivity at a cut off threshold of >1.937 with AUC 69% Table 2-7, Figure 2-15.

Salivary Biomarker	Cut-off	Specificity (%)	Sensitivity (%)	AUC (%)	P value
Composite Marker (HNP1-3+S100A8)	>1.937	92	84	96	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-7: Showing the different parameters of the composite marker (HNP1-3+S100A8) cut-off point concentration that significantly differentiate between Gingivitis and Chronic Periodontitis.

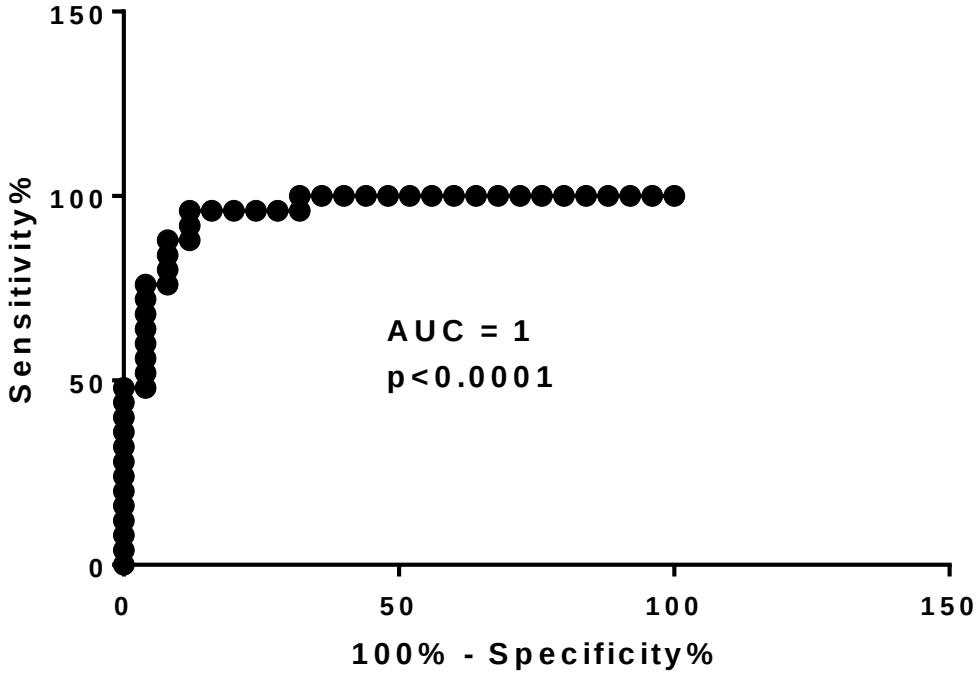

Figure 2-15: ROC curve for the combined normalised values for HNP1-3 and S100A8 using cut-offs 3.745 µg/ml for HNP1-3 and 191.3 ng/ml for S100A8.

Unpaired t-test analysis of the combined (normalised) new sets of data showed that the composite biomarker was significantly lower in gingivitis when compared to chronic periodontitis (p< 0.0001)

Figure 2-16.

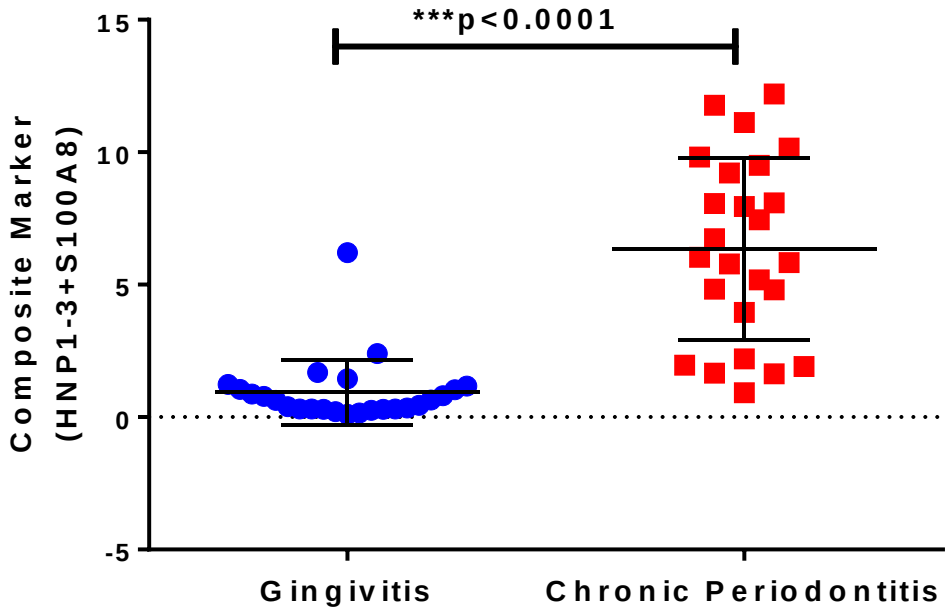

Figure 2-16: Vertical Scatter plot of the summed normalized values of HNP1-3 and S100A8 showing statistically significant higher mean values in chronic periodontitis, using unpaired t-test (two-tailed p value)(p< 0.0001).

Using the second technique (APVT) of combining biomarkers (described before) every sample was assigned as negative or positive according to the pre-chosen cut-off points (3.675 µg/ml for HNP1-3 and 201.3 ng/ml for S100A8) and then added together. The combined sample was positive if either of the markers was positive, and negative only when both markers were negative Table 2-8. Using the Fisher`s exact test contingency table analysis by GraphPad prism software, gave a high specificity of 88.89% and sensitivity of 95.65% Table 2-9.

	Negative	Positive
Gingivitis	22	3
Chronic Periodontitis	1	24

Table 2-8: A contingency table of the combined (HNP1-3 & S100A8) for cases with Gingivitis and Chronic Periodontitis (based on predetermined cut-off points).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(HNP1-3+S100A8)	88.89	95.65	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-9: Showing the specificity and sensitivity of the combined biomarker (HNP1-3 & S100A8) using Fisher`s exact test.

Using this technique for combining biomarkers we were able to detect three CP cases more than either biomarker individually. More specifically the combined marker detected three cases with chronic periodontitis more than HNP1-3 alone, and four cases more than S100A8 alone.

(MMP-8+HNP1-3) Composite Biomarker

A total of 81 cases were available; 40 gingivitis and 41 chronic periodontitis cases with complete AMPs data for MMP-8 & HNP1-3. . As shown earlier in this chapter 2.5.1 that the thresholds were > 82.73 ng/ml for MMP-8 and > 3.675 µg/ml for HNP1-3.

Using the first method (NDM) for combining biomarkers (described before) the composite biomarker (MMP-8+HNP1-3) was able to significantly differentiate between gingivitis and chronic periodontitis with 97.50% specificity and 80.49% sensitivity at a combined threshold of >2.540 with AUC 94% Table 2-10,

Figure 2-17.

Salivary Biomarker	Cut-off	Specificity (%)	Sensitivity (%)	AUC (%)	P value
Composite Marker (MMP-8+HNP1-3)	> 2.540	97.50	80.49	94	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-10: Showing the different parameters of the composite marker (MMP-8+HNP1-3) cut-off point concentration that significantly differentiate between Gingivitis and Chronic Periodontitis.

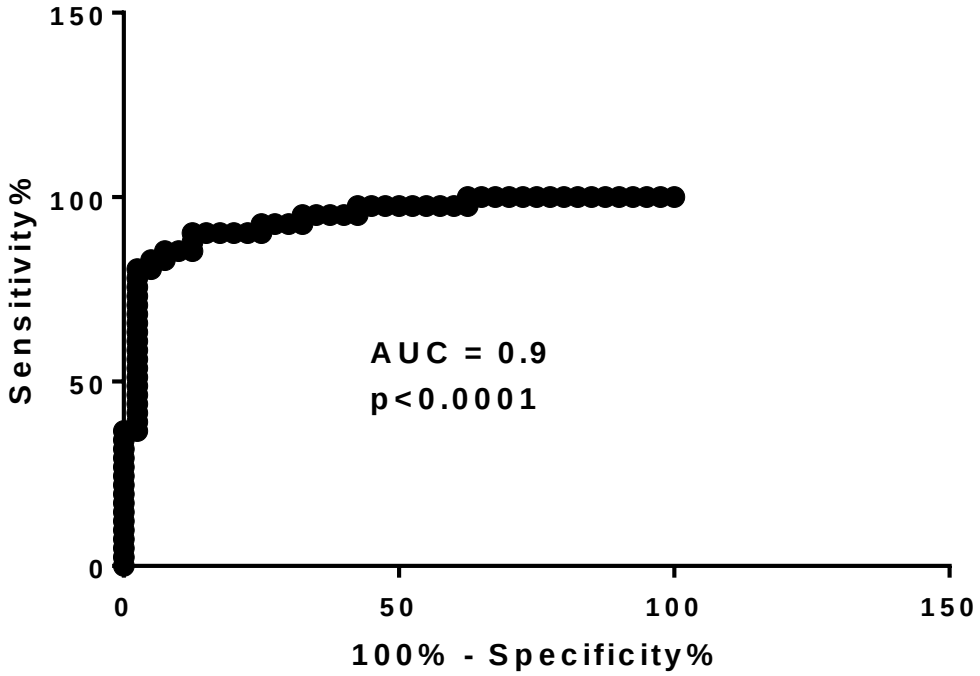

Figure 2-17: ROC curve for the combined normalised values for MMP-8 and HNP1-3 using cut-offs 82.73 ng/ml for MMP-8 and 3.675 µg/ml for HNP1-3.

Unpaired t-test analysis for the combined new sets of data (normalised) showed that the composite biomarker was significantly lower in gingivitis when compared to chronic periodontitis ($p < 0.0001$)

Figure **2-18**.

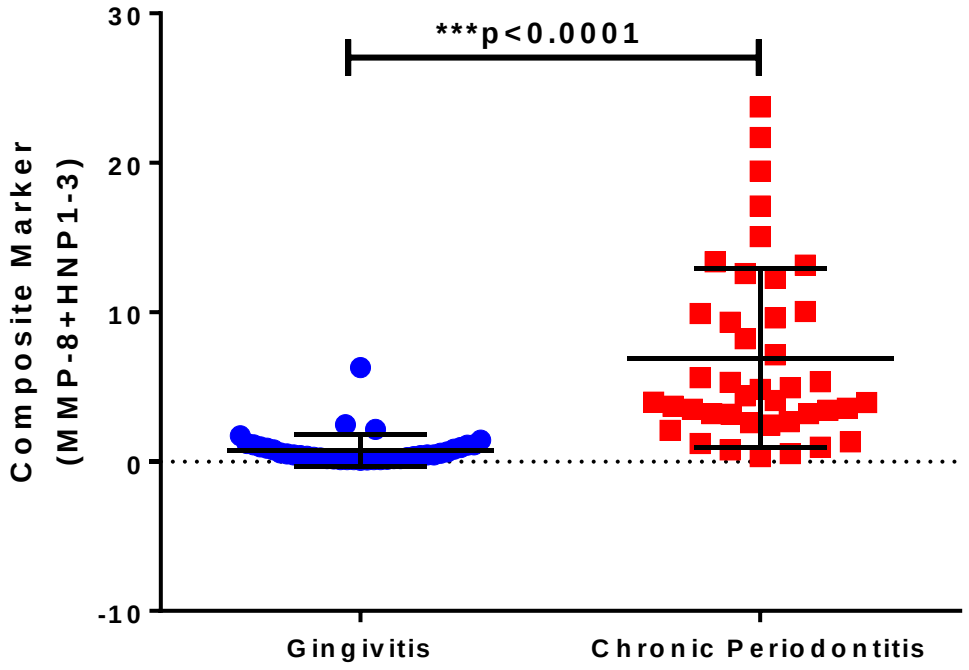

Figure 2-18: Vertical Scatter plot of the summed normalized values of MMP-8 and HNP1-3 showing statistically significant higher mean values in chronic periodontitis, using unpaired t-test (two-tailed p value)(p< 0.0001).

Using the second technique (APVT) of combining biomarkers (described before); every sample was assigned as negative or positive according to the pre-chosen cut-off points (82.73 ng/ml for MMP-8 and 3.675 µg/ml for HNP1-3) and then added together Table 2-11. Using Fisher's exact test contingency table analysis gave both high specificity of 94.87% and sensitivity of 90.48% Table 2-12.

	Negative	positive
Gingivitis	38	2
Chronic Periodontitis	4	37

Table 2-11: A contingency table of the combined (MMP-8 & HNP1-3) for cases with Gingivitis and Chronic Periodontitis (based on predetermined cut-off points).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(MMP-8+HNP1-3)	94.87	90.48	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-12: Showing the specificity and sensitivity of the combined biomarker (MMP-8 & HNP1-3) using Fisher`s exact test.

Using this technique for combining biomarkers was not superior to using individual biomarker in detecting CP cases, as both biomarkers MMP-8 and HNP1-3 were able to detect the same number of cases (37 CP cases) individually and as this composite marker.

(MMP-8+S100A8) Composite Biomarker

A total of 50 cases were available, 25 gingivitis and 25 chronic periodontitis cases with complete AMPs data for MMP-8 & S100A8. As shown earlier in this chapter 2.5.1 the thresholds were > 82.73 ng/ml for MMP-8 and > 201.3 ng/ml for S100A8.

Using the first method (NDM) for combining biomarkers (prescribed before); by constructed ROC curve for the new set of normalised summed data, the composite biomarkers (MMP-8+S100A8) was able to significantly differentiate between gingivitis and chronic periodontitis with 100 % combined specificity and 92 % combined sensitivity at a combined threshold of >1.924 with AUC 98 % Table 2-13, Figure 2-19.

Salivary Biomarker	Cut-off	Specificity (%)	Sensitivity (%)	AUC (%)	P value
Composite Marker (MMP-8+S100A8)	> 1.924	100	92	98	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-13: Showing the different parameters of the composite marker (MMP-8+S100A8) cut-off point concentration that significantly differentiate between Gingivitis and Chronic Periodontitis.

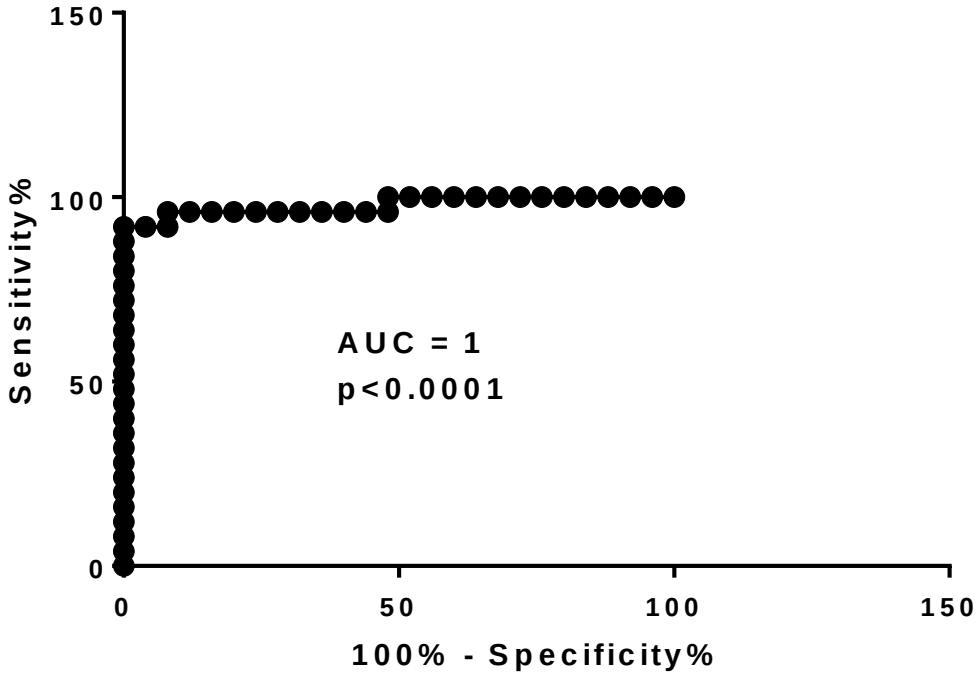

Figure 2-19: ROC curve for the combined normalised values for MMP-8 and S100A8 using cut-offs 82.73 ng/ml for MMP-8 and 201.3 ng/ml for S100A8.

Unpaired t-test analysis for the combined new sets of data (normalised) showed that the composite biomarker was able to significantly differentiate gingivitis from chronic periodontitis ($p< 0.0001$) Figure 2-20.

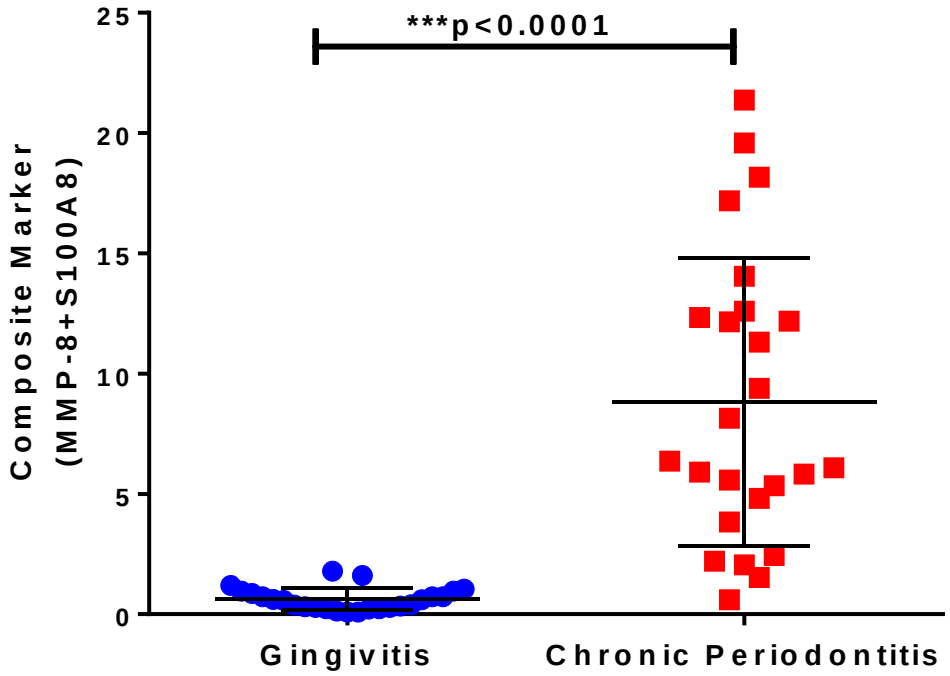

Figure 2-20: Vertical Scatter plot of the summed normalized values of MMP-8 and S100A8 showing statistically significant higher mean values in chronic periodontitis, using unpaired t-test (two-tailed p value)(p< 0.0001).

Using the second technique (APVT) of combining biomarkers (described before) every sample was assigned as negative or positive according to the pre-chosen cut-off points (82.73 ng/ml for MMP-8 and 201.1 ng/ml for S100A8) and then added together. The combined sample was scored positive if either of the markers was positive and negative only when both markers were negative Table 2-14. Using the Fisher`s exact test contingency table analysis by GraphPad prism software, gave both high specificity of 92.31% and sensitivity of 95.83% Table 2-15.

	Negative	Positive
Gingivitis	23	2
Chronic Periodontitis	1	24

Table 2-14: A contingency table of the combined (MMP-8 & S100A8) for cases with Gingivitis and Chronic Periodontitis (based on predetermined cut-off points).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(MMP-8+S100A8)	92.31	95.83	**<0.0001***

***Statistical significance (p<0.05)**

Table 2-15: Showing the specificity and sensitivity of the combined biomarker (MMP-8 & S100A8) using Fisher`s exact test.

Using this technique for combining biomarkers we were able to detect three cases with Chronic Periodontitis more than either biomarker individually. More specifically the combined marker detected four cases with chronic periodontitis more than S100A8 alone, and three cases more than MMP-8 alone.

Overall comparison of the results of the two methods of combining biomarkers reveals that the first method (NDM) was slightly more specific but less sensitive in detecting disease/healthy cases than the second (APVT). A summary table of the biomarkers combination results using the two different methods of combination are shown in Table 2-16.

Composite Biomarker	No. of Cases	NDM Specificity (%)	NDM Sensitivity (%)	APVT Specificity (%)	APVT Sensitivity (%)
(MMP-8+HNP1-3+S100A8)	50	**96**	92	88.89	**95.65**
(HNP1-3+S100A8)	50	**92**	84	88.89	**95.65**
(MMP-8+HNP1-3)	81	**97.50**	80.49	**94.87**	90.48
(MMP-8+S100A8)	50	**100**	92	92.31	**95.83**

Table 2-16: Summary of the composite biomarkers comparing the results of the two combining methods.

2.5.3 The application of pre-determined cut-off points for MMP-8, HNP1-3 & S100A8 to an independent cohort to diagnose periodontal disease

The results above suggest that salivary MMP-8, HNP1-3 and S100A8 individually and as a composite biomarker have the ability to differentiate between gingivitis and chronic periodontitis with fairly high specificity and sensitivity. In order to validate this observation, and particularly to test the cut-off points for a positive test determined in the previous study, we tested the ability of these biomarkers to detect periodontal disease in an independent cohort of subjects which had been collected on the basis of their diabetic status and thus were of continuously variable periodontal disease status.

The basic features of the samples of this cohort are shown in Table 2-17.

	Gingivitis/Mild Periodontitis	Moderate Periodontitis	Severe Periodontitis	Total number
Non diabetic	60	13	6	79
Well-controlled diabetic	12	6	1	19
Poorly-controlled diabetic	59	29	7	95
Total number	131	48	14	**193**

Table 2-17: Showing the distribution of the independent cohort subjects according to the diabetic status and periodontal diagnosis according to the case definition.

Within this cohort, the application of the predetermined cut-off points for HNP1-3 (> 3.675 µg/ml) detected periodontitis with 87.50% specificity and 33.77% sensitivity and for S100A8 (> 201.3 ng/ml) 77.63% specificity, 26.09 % sensitivity Figure 2-21, Figure 2-22. While for MMP-8 the application of pre-chosen threshold (>82.73 ng/ml) detected periodontitis with 80.15% specificity and 37.10% sensitivity Figure 2-23.

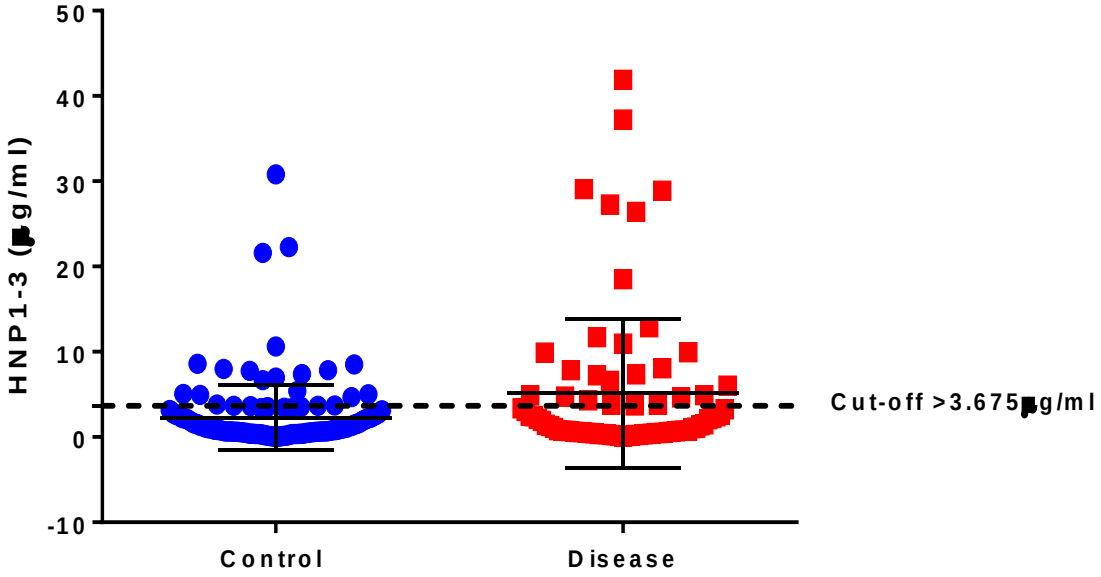

Figure 2-21: Vertical scatter plot graph showing the application of HNP1-3 pre-chosen cut-off (>3.675µg/ml) to the independent cohort.

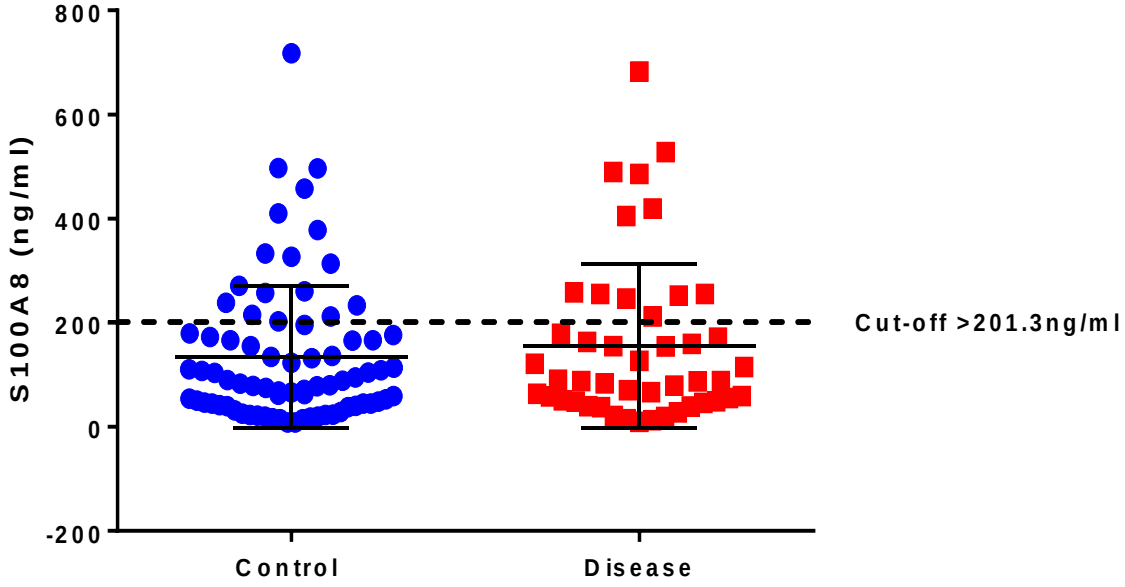

Figure 2-22: Vertical scatter plot graph showing the application of S100A8 pre-chosen cut-off (>201.3ng/ml) to the independent cohort.

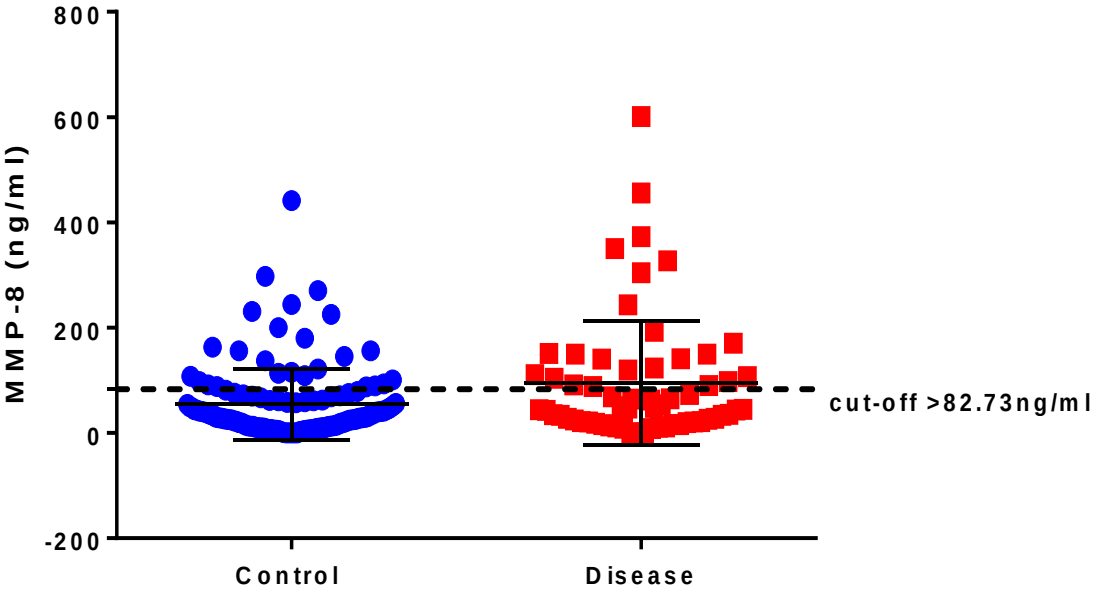

Figure 2-23: Vertical scatter plot graph showing the application of MMP-8 pre-chosen cut-off (>82.73ng/ml) to the independent cohort.

Using the first technique (NDM) described before to combine all three biomarkers (by normalising the data to the chosen cut-off); a combined cut off point for (MMP-8 + HNP1-3 + S100A8) (> 2.512) showed 74.55 % specificity, with 25.81 % sensitivity Figure 2-24.

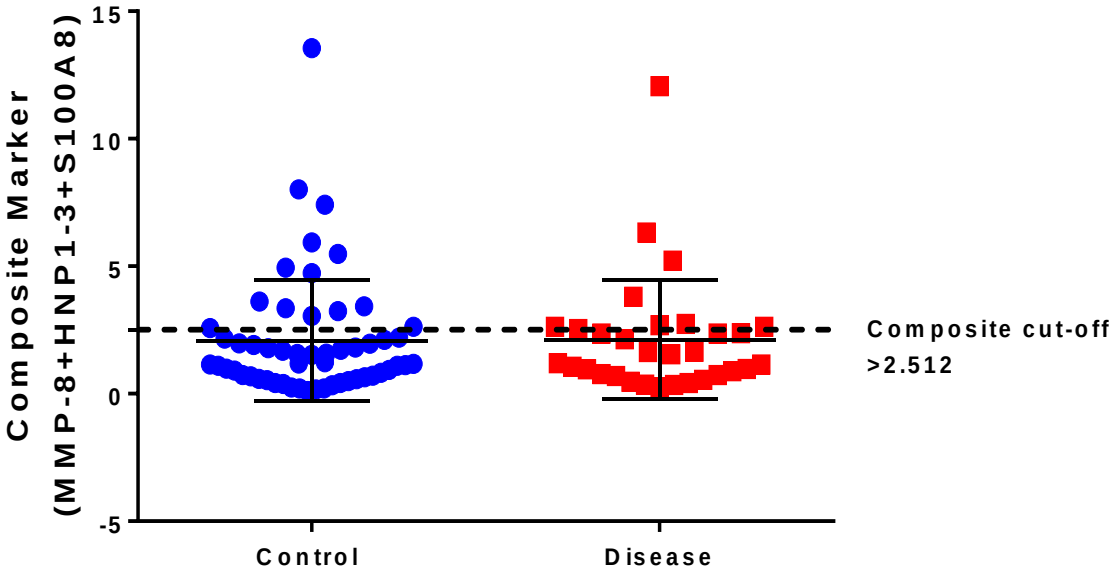

Figure 2-24: Vertical scatter plot graph showing the application of the composite (MMP-8+HNP1-3+S100A8) pre-chosen cut-off (>2.512) to the independent cohort.

Using the second way of combining biomarkers (APVT – any positive value test) Table 2-18 gave 42.42 % specificity and 67.92 % sensitivity Table 2-19, which is in contrast to the results obtained using the first way of combining biomarkers.

	Negative	Positive
Gingivitis	36	19
Chronic Periodontitis	17	14

Table 2-18: A contingency table of the combined (MMP-8 + HNP1-3 + S100A8) for cases with Gingivitis and Chronic Periodontitis of the independent cohort (using APVT).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(MMP-8+HNP1-3+S100A8)	42.42	67.92	0.4

Table 2-19: Showing the specificity and sensitivity of the combined biomarker (MMP-8 + HNP1-3 + S100A8) of the independent cohort using Fisher`s exact test.

For the composite biomarker (HNP1-3 + S100A8) using the combined analytes by NDM method the application of the predetermined cut-off point (1.937) was able to detect periodontitis with 78.67 % specificity and 23.91 % sensitivity Figure 2-25.

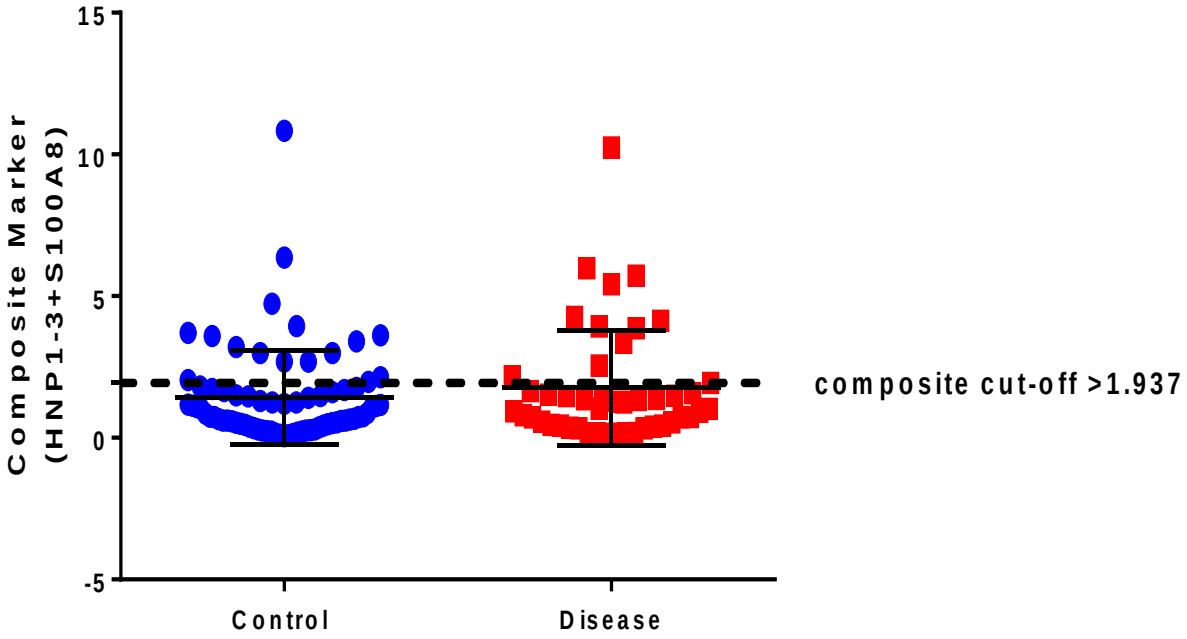

Figure 2-25: Vertical scatter plot graph showing the application of the composite (HNP1-3+S100A8) pre-chosen cut-off (>1.937) to the independent cohort.

While by using APVT technique for combining HNP1-3 + S100A8 Table 2-20 gave a combined specificity of 46.15 % and sensitivity of 65.85 % Table 2-21.

	Negative	Positive
Gingivitis	54	21
Chronic Periodontitis	28	18

Table 2-20: A contingency table of the combined (HNP1-3 + S100A8) for cases with Gingivitis and Chronic Periodontitis (based on predetermined cut-off points).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(HNP1-3+S100A8)	46.15	65.85	0.2

Table 2-21: Showing the specificity and sensitivity of the combined biomarker (HNP1-3 + S100A8) using Fisher`s exact test.

Moreover, the application of the pre-chosen threshold for the composite marker (MMP-8 + HNP1-3) (>2.540), by NDM technique, to the independent cohort detected periodontitis with 86.26 % combined specificity and 25.81 % combined sensitivity Figure 2-26.

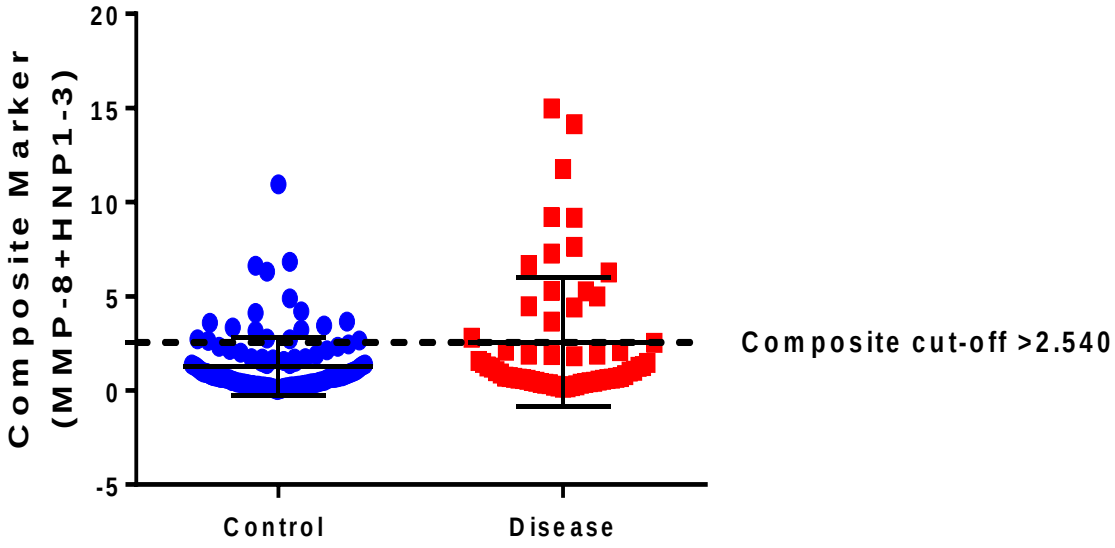

Figure 2-26: Vertical scatter plot graph showing the application of the composite (MMP-8+HNP1-3) pre-chosen cut-off (>2.540) to the independent cohort.

When the APVT technique was used Table 2-22, the analysis to the independent cohort according to the predetermined cut-off gave 47.37 % specificity and 74.26 % sensitivity Table 2-23.

	Negative	positive
Gingivitis	101	30
Chronic Periodontitis	35	27

Table 2-22: A contingency table of the combined (MMP-8 + HNP1-3) for cases with Gingivitis and Chronic Periodontitis (based on APVT).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(MMP-8+HNP1-3)	47.37	74.26	**0.004***

***Statistical significance (p<0.05)**

Table 2-23: Showing the specificity and sensitivity of the combined biomarker (MMP-8+HNP1-3) of the independent cohort using Fisher`s exact test.

In addition, application of composite markers MMP-8 + S100A8 (>1.924), by NDM technique, to the independent cohorts was able to detect periodontitis with combined 81.82 % specificity and 29.03 % sensitivity Figure 2-27.

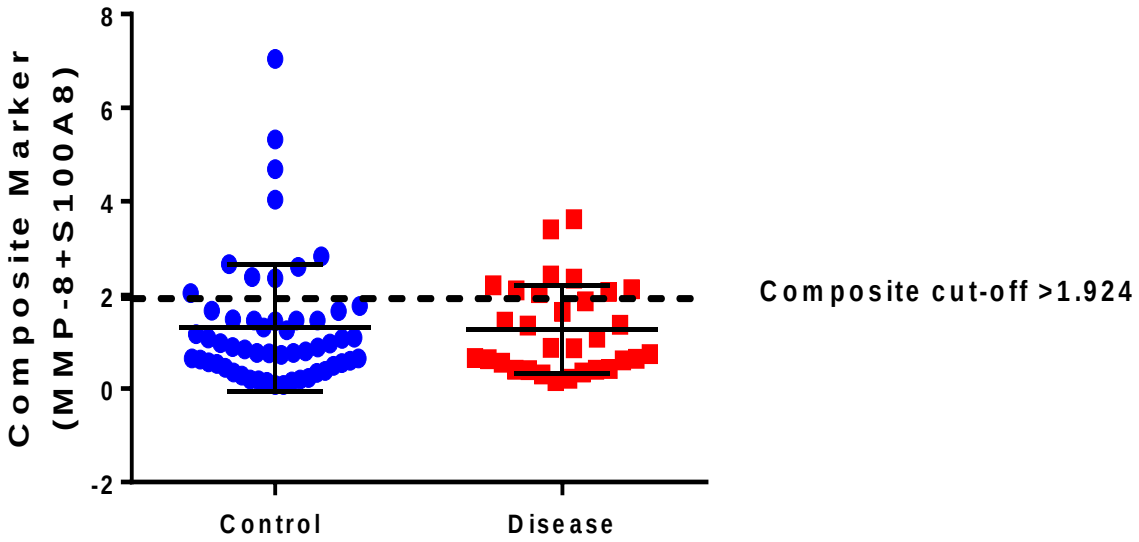

Figure 2-27: Vertical scatter plot graph showing the application of the composite (MMP-8+S100A8) pre-chosen cut-off (>1.924) to the independent cohort.

However when using the APVT technique Table 2-24, the analysis gave 41.38 % specificity and 66.67 % sensitivity Table 2-25.

	Negative	positive
Gingivitis	38	17
Chronic Periodontitis	19	12

Table 2-24: A contingency table of the combined (MMP-8 + S100A8) for cases with Gingivitis and Chronic Periodontitis of the independent cohort (By APVT).

Biomarker	Specificity (%)	Sensitivity (%)	P value
(MMP-8+S100A8)	41.38	66.67	0.5

Table 2-25: Showing the specificity and sensitivity of the combined biomarker (MMP-8+S100A8) of the independent cohort using Fisher`s exact test.

A summary of the results for individual and combined biomarkers is shown in Table 2-26.

Biomarker	Specificity (%)	Sensitivity (%)	P value
HNP1-3	87.50	33.77	0.08
S100A8	77.63	20.09	0.6
MMP-8	80.15	37.10	**0.01***
(MMP-8+HNP1-3+S100A8)	74.55	25.81	0.7
(HNP1-3+S100A8)	78.67	23.91	0.5
(MMP-8+HNP1-3)	86.26	25.81	**0.01***
(MMP-8+S100A8)	81.82	29.03	0.6

***Statistical significance (p<0.05)**

Table 2-26: Summary of the application of pre-determined cut-offs results, for individual and combined biomarkers, into the independent cohort.

2.5.4 The effects of diabetes mellitus on biomarker status

Although previous pilot data in our lab had suggested that there were no differences in salivary biomarkers due to the diabetic status of the subjects we tested this further by 2-way ANOVA using periodontal disease status (healthy, gingivitis / mild periodontitis, and moderate to severe periodontitis) and diabetic status (no diabetes, well–controlled diabetes and poorly controlled diabetes) as explanatory variables. Bonferroni Post-Tests were used to identify significant differences between each data category.

The results for MMP-8 are shown in Table 2-27 and Figure 2-28. There was a significant effect of diabetes and periodontal status on the concentrations of MMP-8 and a significant interaction between periodontal disease and diabetes.

The effect of Diabetes was not significant in the absence of any periodontal disease.

Two-way ANOVA for MMP-8		
Source of Variation	**% of total variation**	**P value**
Interaction	5.36	**0.02***
Diabetes	6.13	**0.001***
Perio	9.69	**< 0.0001***
Bonferroni post-tests		
No Diab vs Well controlled		
No Perio		> 0.05
Mild Perio		**< 0.01***
severe		**< 0.05***
No Diab vs poorly controlled		
No Perio		> 0.05
Mild Perio		**< 0.01***
severe		**< 0.05***

***Statistical significance (p<0.05)**

Table 2-27: Summary Results of 2-way ANOVA for MMP-8.

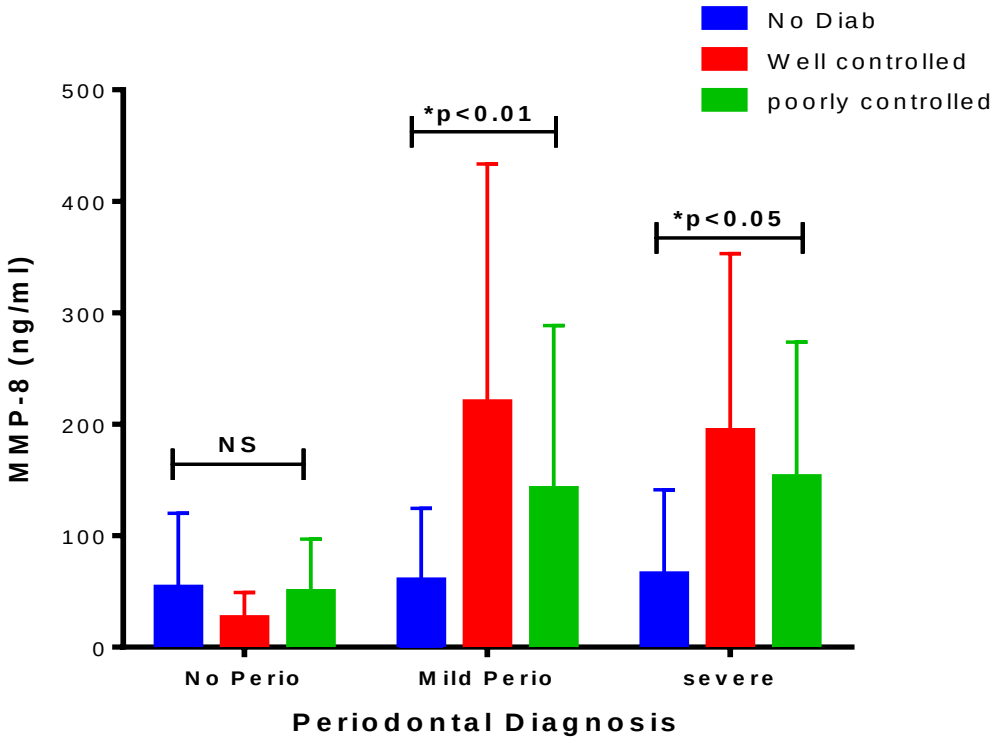

Figure 2-28: Bar graph illustrates the significant influence of DM on MMP-8 in mild and moderate-severe periodontitis.

The results for HNP are shown in Table 2-28 and Figure 2-29. There was no significant effect of diabetes or periodontal status on the concentrations of HNP and no significant interaction between periodontal disease and diabetes.

Two-way ANOVA for HNP		
Source of Variation	**% of total variation**	**P value**
Interaction	0.09	1
diabetes	0.04	1
Perio	0.25	0.8

Table 2-28: Summary Results of 2-way ANOVA for HNP.

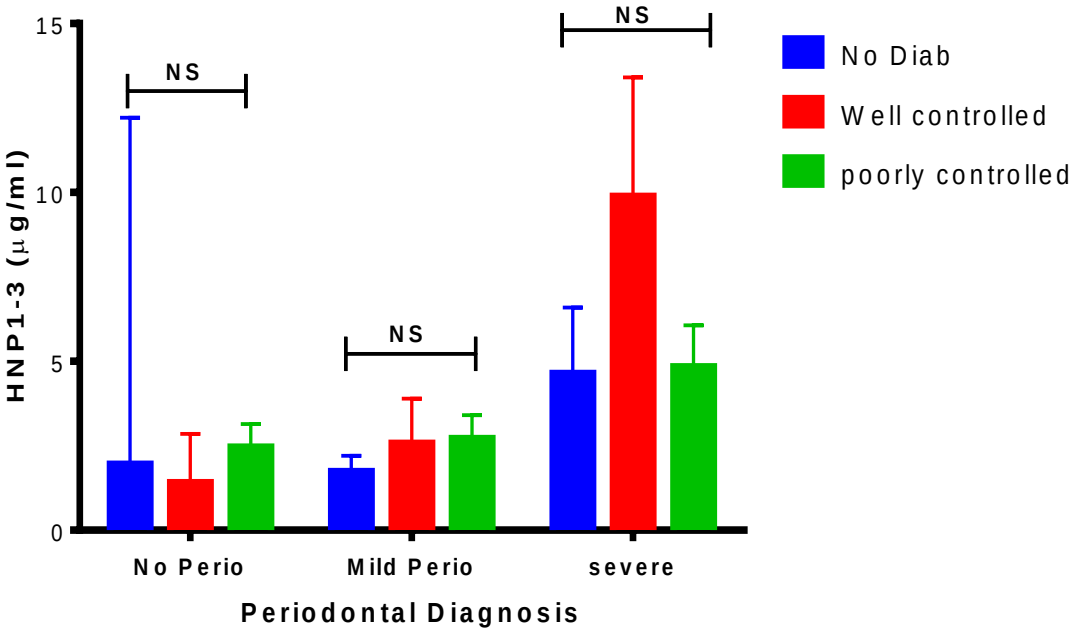

Figure 2-29: Bar graph illustrates the non-significance influence of DM on HNP1-3 in different periodontal diagnoses.

The results for S100A8 are shown in Table 2-29 and Figure 2-30. Overall there was a significant effect of periodontal status on the concentrations of S100 and a significant interaction between periodontal disease and diabetes, but no direct significant of diabetes. Bonferroni post-tests were all non-significant.

Two-way ANOVA of S100a8		
Source of Variation	**% of total variation**	**P value**
Interaction	5.61	**0.02***
Diabetes	0.84	0.4
Periodontal disease	4.28	**0.01***
Bonferroni post-tests		
No Diab vs Well controlled		
No Perio		P > 0.05
Mild Perio		P > 0.05
severe		P > 0.05
No Diab vs poorly controlled		
No Perio		P > 0.05
Mild Perio		P > 0.05
Severe		P > 0.05

***Statistical significance (p<0.05)**

Table 2-29: Summary Results of 2-way ANOVA for S100.

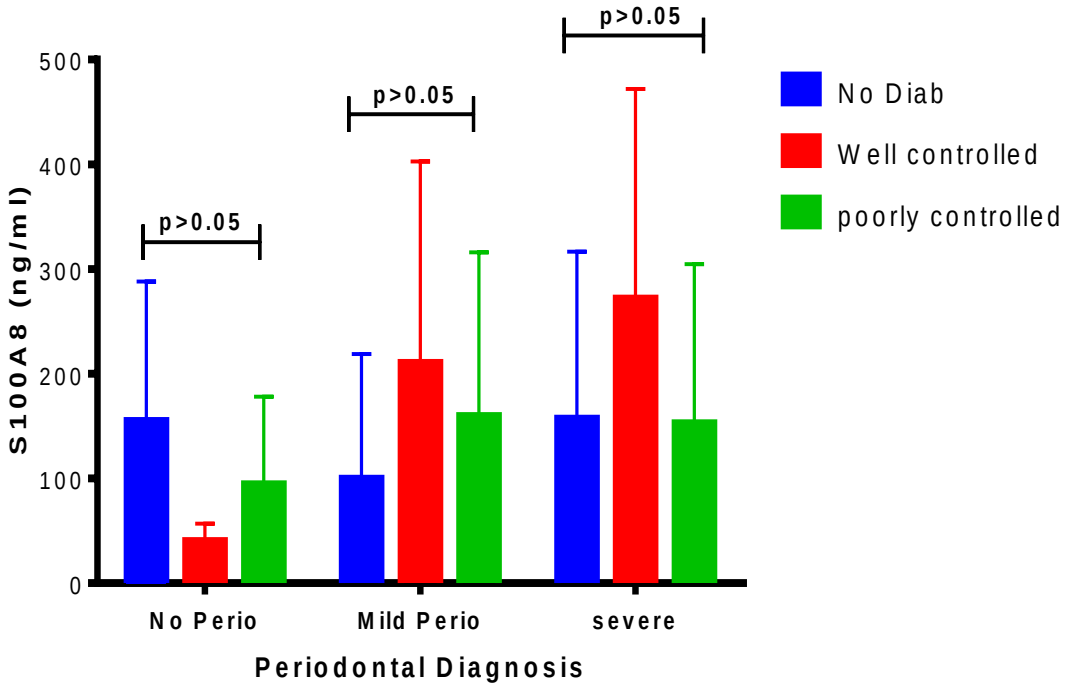

Figure 2-30: Bar graph illustrates the effects of DM on S100A8 in different periodontal diagnoses.

2.6 Discussion

The main objectives of this study were to verify and establish a diagnostic threshold for a group of salivary biomarkers (MMP-8, HNP1-3, S100A8 and LL-37) and to test the validity of the diagnostic utility of these biomarkers. This involved the determination of optimal cut off points using samples of known periodontal status, and then the application of these previously determined cut-off points to an independent cohort of subjects.

The independent group was a particularly good test for this study as the participants were recruited based on their diabetic and not periodontal status. This cohort comprised of relatively young individuals (mean age of 36 years) who were non-smokers for at least 5 years, and approximately 2/3rds with type I diabetes. However, most of them had only mild periodontal disease.

In the first series of experiments to establish cut off points for salivary AMPs and to compare these results with salivary MMP-8, our findings firstly confirmed previous results in our lab of the potential diagnostic utility of HNP1-3 and S100A8. The ELISA analysis for the salivary antimicrobial peptides showed that HNP1-3 and S100A8 concentrations could differentiate between gingivitis and chronic periodontitis with high specificity (around 90%) and around 75% sensitivity. This is similar to the findings of previous studies for S100A8 which have reported that their levels in GCF and saliva are elevated in periodontitis (Kido et al., 1999; Kojima et al., 2000; Ramseier et al., 2009). Likewise, α-defensins (HNP1-3) GCF levels have been reported to be higher in chronic and aggressive periodontitis than healthy controls (Puklo et al., 2008), although other studies have reported that there was no significant difference in the HNP1-3 concentrations between diseased and healthy periodontium (Lundy et al., 2005; Turkoglu et al., 2010).

On the other hand, LL-37 concentrations were not significantly different between disease and control groups, which is in contrast with the findings of Turkoglu and colleagues (Türkoğlu et al., 2009) of elevated levels of LL-37 in patients with chronic periodontitis as well as our previous studies that showed LL-37 was specifically elevated in Aggressive Periodontitis (Mulli, 2012). Subsequently, LL-37 was thus not investigated further in our subsequent studies.

When comparing our results for HNPs and S100 with those for MMP-8, we found that MMP-8 also was able to discriminate between gingivitis and periodontitis (chronic and aggressive) with both high specificity and sensitivity. The results with MMPs showed a slightly improved sensitivity compared to the AMPs. This finding is in keeping with reports of MMP-8 a reliable marker for periodontal disease as it showed significant high levels in chronic periodontitis compared to healthy controls (Miller et al., 2006; Todorovic et al., 2006). However, MMP-8 was unable to discriminate chronic from aggressive periodontitis in our study that goes well with other study`s finding of no marker in saliva or GCF that could distinguish between chronic and aggressive periodontitis (Loos and Tjoa, 2005).

In addition, our preliminary results regarding aggressive periodontitis showed that only HNP1-3 and MMP-8 might has the ability to discriminate between gingivitis and aggressive periodontitis cases. However, none of them was able to differentiate between chronic and aggressive periodontitis case.

Combining more than one biomarker may significantly improve the diagnostic utility when compared with single biomarker tests (Gursoy et al., 2011; Ramseier et al., 2009). All biomarkers combinations that we have performed gave exceptionally high specificity and high sensitivity; with the best combination being MMP-8 + S100A8 where it was able to detect periodontitis cases with 100 % specificity and 92 % sensitivity. However, combining HNP1-3 with any of biomarkers MMP-8 or S100A8 did not further improve the diagnostic utility of the test compared with combining MMP-8 and S100A8 only.

We used 2 different methods to combine the biomarker data to produce a composite biomarker test, the Normalised Data Method (NDM) which allowed summation of different markers by normalising their values first and the Any Positive Value Test (APVT) where by a positive value for any single biomarker was considered as a positive composite biomarker value. Both methods produced rather similar results although the APVT was somewhat less specific. This is perhaps not surprising given the wide variation of biomarker levels between individuals even in control samples.

We then tested these findings on the independent cohort of samples to test the external validity of the results. This was quite a stringent test as in contrast to the initial cohort of samples, which had been collected with discrete periodontal diagnoses, the second cohort had been collected on the basis of their diabetic

status and their periodontal status was continuously variable. In addition there were relatively low levels of periodontitis overall in this cohort. The application of pre-determined thresholds, either individual or combined cut-offs, were able to detect periodontitis with specificity of between around 75 – 85 % but with very low sensitivity. Combination tests using the NDM method tended to be superior to the individual markers alone. Interestingly, using the APVT technique for combining biomarkers had improved the sensitivity but at the expense of specificity.

Although a specificity of around 80+% might potentially be of some use clinically in some situations, such as positive identification of “cases” in epidemiological studies, these results do not suggest overall that these tests, even in combinations, would be useful without additional either clinical or biomarker data.

Given the excellent results obtained with the original cohort studies, some other explanations might help to partially explain the results of the validation study. Most importantly we tested the effects of diabetes on the biomarker levels. In previous pilot studies in our lab we had not seen any evidence of differences between groups of different diabetic status. However here we analysed the cohort allowing both for periodontal and diabetic status at the same time by 2-way ANOVA. This analysis showed clearly that MMP-8 levels were elevated in diabetics with mild or more severe periodontitis. In addition although the effect of diabetes on S100A8 was non-significant, there was significant interaction between the effects of periodontal disease and diabetes which also suggests that diabetes may affect S100A8 in patients with Periodontal Disease. These results suggest that the biomarker tests carried out here may not be valid on subjects with diabetes or of unknown diabetic status. We considered carrying out the analyses on the non-diabetic subjects only, but the numbers of cases of severe periodontitis in the non-diabetic patients were considered too small to be very valid or useful.

Finally the samples used in the validation study were collected in Glasgow by a different research group and although the reported collection method used by them appears the same as our own method this might have affected the samples if there was any variation. These samples were shipped to London by Courier on ice but some had at least partially defrosted in transit.

2.7 Conclusion

Overall the results of the studies in this chapter suggest that MMP-8, HNP1-3 and S100A8 may be useful to identify cases of periodontitis with good specificity and less good sensitivity and may give superior results when combined, particularly using the NDM technique. The validation study suggests that the test may not be useful in patients with diabetes. Additional validation studies would be useful in larger, non-diabetic cohorts to investigate further the use of these biomarkers.

CHAPTER 3- PROGNOSTIC BIOMARKERS OF PERIODONTAL DISEASE: A LONGITUDINAL STUDY

3. Prognostic Biomarkers of Periodontal Disease: A Longitudinal Study

The prediction of patients' response (outcome) to periodontal treatment remains problematic. Due to the complicated nature and multifactorial aetiology of periodontal diseases, it has been difficult to identify markers with diagnostic and prognostic utility. Currently, clinicians rely on certain criteria (particularly clinical findings such as probing pocket depth, bleeding on probing, radiographical assessment of alveolar bone loss and also on general factors such as age, medical history and smoking) that have been used quite subjectively to identify susceptible individuals and active sites. Although these criteria are easy to use, their prognostic utility is very limited (Giannobile et al., 2009). Therefore, the idea of the identification of a novel, reliable, simple and non-invasive diagnostic/prognostic tool for periodontal disease is very interesting and attractive to clinical researchers.

Over recent decades, researchers have targeted oral fluids in order to investigate possible prognostic markers. Obviously, finding a single biomarker to detect or predict periodontal disease is unexpected due to the complexity of periodontal disease. In this matter, the literature is not rich with longitudinal studies looking into changes of local and systemic biomarkers during periodontal treatment.

As already discussed, there can be considerable variations in treatment outcomes between individuals, and poor responses to treatment tend to cluster in a minority of patients. In the last chapter 2 we investigated the diagnostic utility of certain salivary biomarkers, both singly and combined, and showed a significant ability to detect periodontal disease. Therefore, we would like to take this further and investigate the potential prognostic utility of these biomarkers in a longitudinal intervention study of periodontal treatment.

3.1 Aims & Objectives

The specific aims of this longitudinal study intervention were:

1. To test the prognostic capability of the recently identified AMPs (HNP1-3 & S100A8) and MMP-8 individually and in combination on initial outcome of nonsurgical periodontal treatment.
2. To investigate the changes in salivary analytes with time in response to periodontal treatment.
3. To investigate the relationship of salivary analytes to systemic inflammatory markers (CRP & IL-6) in circulating blood, at baseline and following non-surgical periodontal treatment.
4. To investigate if these systemic inflammatory markers (CRP, IL-6) have any prognostic utility.

3.2 Material and Methods

3.2.1 Ethical Approval

The protocol for this study was reviewed and approved by Newcastle and North Tyneside Research Ethics Committee 2 on 21st March 2012, reference number: (12/NE/0122). All the participants signed an informed written consent form.

3.2.2 Inclusion Criteria

The participants (males and females from any ethnicity) included in this study were generally healthy (smokers and diabetics were included), between the ages 18 to 65 years and had chronic periodontitis (see case definition below 3.2.4). All participants were recruited either from patients' waiting list or actively from consultant-led new patient clinics at the Department of Periodontology at Guy`s Dental Hospital. All participants had at least 20 natural teeth.

3.2.3 Exclusion Criteria

Patients were excluded from the study if the patient had:

1. Complicating medical history. Anybody taking medication likely to affect gingival health, including oral contraceptive pills, calcium channel blockers, phenytoin, cyclosporine, and any regular use of anti-inflammatory medication including corticosteroids and NSAIDS.
2. Medical conditions likely to affect gingival health, such as pregnancy.
3. Periodontal treatment within the last 6 months or antimicrobial therapy within the last 3 months.
4. Identifiable blood borne transmissible disease, such as hepatitis.

3.2.4 Case Definitions

Moderate and severe Chronic Periodontitis were defined from the CDC /AAP criteria (Page and Eke, 2007) as below:

a. Moderate Periodontitis:

Patients with two or more interproximal sites with clinical attachment loss of ≥ 4mm occurring at two or more different teeth OR two or more interproximal sites with a probing depth of ≥ 5mm, not on the same tooth.

b. Severe Periodontitis:

Patients with two or more interproximal sites with clinical attachment loss of ≥ 6mm not on the same tooth, and the presence of one or more interproximal sites with a probing depth of ≥ 5mm.

In addition, a clinical diagnosis of chronic periodontitis was made by a consultant.

3.2.5 Study Populations & Protocol

The power calculation for this study was based on comparing the salivary biomarker (MMP-8) levels before and after periodontal treatment using a paired sample t-test. Assuming an effect size of 0.4 with 80% power, the study will require a total sample of 52 to detect the difference at 5% level of significance. The power calculation was carried out using G power version 3.1.5.

The recruited participants were informed and consented in order to be enrolled in the study. As an overview of the protocol, every participant was offered four appointments at the department of Periodontology at Guy`s Dental Hospital. At the first visit they had a thorough periodontal assessment. During second and third visits non-surgical periodontal therapy was carried out (with or without local anaesthesia using both manual and ultrasonic scalers). At the final visit a thorough reassessment was carried out. In addition, they were asked to provide saliva, blood and plaque samples before and after the provided therapy. See Table 3-1 for full description of the protocol.

In more detail, at the first study visit, a full history was recorded from every participant (after they were fully informed and consented), saliva & blood samples were taken and then a full clinical assessment was performed. Smoking status of

the participants was either current smoker (self-reported) or non-smoker (never or ex-smoker). The periodontal assessment (baseline data) included: plaque index (PI= the number of sites with detectable supra-gingival plaque x 100/ the total number of sites per mouth), bleeding index (BI= the number of sites with gingival bleeding on probing x 100/ the total number of sites per mouth), six point probing pocket depth (PPD), gingival recession, tooth mobility and furcation involvement. The BI, PPD and gingival recession were measured using a William's graduated probe. Therefore, readings > 10mm in probing pocket depth were omitted from analysis. The number of these sites (>10mm PPD) were small; a total of 15 sites in eight subjects.

At the second study visit, three plaque samples were taken from three deep sites of > 4mm probing pocket depth, using paper points and placed in Eppendorf tubes and stored at -80°C until time for future analysis. Whenever possible sites from both anterior and posterior, and upper and lower teeth were sampled, depending on distribution of deep sites. Then half-mouth supra- & sub-gingival scaling using both manual and ultrasonic instrumentations was performed under local anaesthetic. At the third study visit, non-surgical root surface debridement was performed in the remaining quadrants, and full mouth polishing was performed and further OHI was given. Eight to ten weeks later (after completion of the treatment), participants were seen for saliva, blood and plaque sampling and reassessment of all clinical variables assessed at baseline.

In addition, oral hygiene instruction was given and plaque & bleeding scores were performed at every visit of the study. All teeth were included except third molars. Initially in the study, all demographic data and clinical measurements were recorded on the Clinical Study Record Form (see Appendix 5.2.3), while later all the data were recorded electronically using clinical information system (SALUD software system, Ireland) for dental schools and hospitals.

	Visit (1)	Visit (2)	Visit (3)	Visit (4)
Visit duration	60 min	90 min	90 min	60 min
Sample collection (saliva & blood)	✓	X	X	✓
Sample collection (Plaque)	X	✓	X	✓
(OHI, PI & BI)*	✓	✓	✓	✓
Thorough Periodontal Assessment	✓	X	X	✓
Non-surgical Periodontal treatment	X	✓	✓	X

***OHI: Oral Hygiene Instructions, PI: Plaque Index, BI: Bleeding Index.

Table 3-1: Showing the work to be done in every visit of this longitudinal study.

Intra-examiner reproducibility

A total of eight participants were randomly selected in order to re-measure both probing pocket depth (PPD) and recession (Rec) of half-mouth (two quadrants), at the same visit (either at baseline or reassessment visits). The side selection was random; four participants were selected for the right side and four for the left side. All the readings were carried out by the same clinical researcher (BK). A total of 560 sites were included in the reproducibility study.

3.2.6 Sample Acquisition

3.2.6.1 Whole saliva sample collection and processing

Unstimulated whole saliva was collected twice from every participant; at the first and last study visits, before starting the clinical periodontal examination. Subjects were asked to passively drool into a pre-weighed sterile plastic tube for 5 minutes, then the collected tubes were transferred directly into an ice box to be carried to the lab at Guy`s Hospital where all the samples were processed and stored. The processing procedure of the collected saliva started with weighing the tubes again (to get the net saliva volume), vortexing them for 20-30 seconds and centrifuging the samples at 3000 rpm at (4°C) for 15 minutes. The processed saliva was aliquotted into small Eppendorf Tubes® (500µl capacity), labelled and logged-in using a sample tracking software (Pro-curo Software Ltd, UK) and Brady System hardware, then stored at - 80°C in freezers until the time for analysis.

3.2.6.2 Blood sample collection and processing

A volume of 5 ml of venous blood from the antecubital fossa was collected at the first and last study visits before commencing the periodontal clinical examination. Once the blood drained into gold cap Vacutainer® tubes, it was allowed to clot for 30 minutes then centrifuged at 2900 rpm at 20°C for 10 minutes. The serum was aliquotted into small Eppendorf Tubes® (500µl capacity), labelled and logged-in using a sample tracking software (Pro-curo Software Ltd, UK) and Brady System hardware. The samples were stored at - 80°C in freezers until the time for analysis.

3.2.7 Saliva & Serum Analysis using ELISAs

3.2.7.1 ELISAs for Saliva Samples

ELISAs for MMP-8, HNP1-3, and S100A8 were carried out on all saliva samples collected from every participant before and after non-surgical periodontal treatment. To quantify MMP-8, the human total MMP-8 DuoSet kit (R & D Systems, inc. USA) was used to determine the concentrations of MMP-8 according to the manufacturer's instructions. HNP1-3 proteins commercial kit for HNP1-3 (Hycult biotechnology b.v The Netherlands) was used, while Circulex

S100A8 (Human) ELISA kit (CYclex Co., Ltd.Terasawaoka, Japan) commercial kit was used to quantify S100A8 protein according to manufacturer's instructions (See Appendix 5.1.1 for detailed protocols).

The reported minimum detectable concentrations of HNP1-3 and S100A8 were 156 pg/ml and 43.4 pg/ml respectively, and the coefficient of variance (intra-assay precision) ranges were <10% and between 3.6%-4.1% respectively.

In brief, saliva samples and ELISA reagents were thawed at room temperature. The samples for saliva were diluted at an optimized dilution factor of X 300 for MMP-8 (Serial Dilution D1= 1:10, D2=1:30), X 5000 for HNP1-3 (Serial Dilution D1=1:250, D2=1:20), and x 100 for S100A8 (with second reading of the plates at 405nm for high concentration samples). For S100A8, some saliva samples showed higher optical density readings than the highest standard (S1), therefore were repeated with higher dilution factor (X250). Both standards and samples were run in duplicates. Colorimetric reading was done using Dynex Revelation® 4.24 at 450nm. Standard curves were plotted using Microsoft Windows® Excel 2010 spreadsheet software.

3.2.7.2 ELISAs for Serum Samples

ELISAs for CRP and IL-6 were carried out on all serum samples collected from every participant before and after the non-surgical periodontal treatment. To quantify CRP, the high sensitivity CRP kit (Kalon Biological Ltd, UK) was used to determine the concentrations of CRP according to the manufacturer's instructions. High sensitivity Human IL-6 commercial kit for IL-6 (Quantikine® HS ELISA, R&D Systems, Inc. USA) was used according to the manufacturer's instructions. (See Appendix 5.2.3 for detailed protocols).

The minimum detectable dose of CRP was 0.20 mg/L and for IL-6 ranged from 0.016-0.110 pg/ml, and a coefficient of variation (Intra-assay precision) for CRP between 5.2% and 15% and for IL-6 between 6.9% and 7.8%.

In brief, serum samples and ELISA reagents were thawed at room temperature. The samples for serum were diluted at an optimized dilution factor of (X 1000) for CRP (Serial Dilution D1= 1:50, D2=1:20). While for IL-6, the serum samples were run neat (undiluted). Both standards and samples were run in duplicates.

Colorimetric reading was done using Dynex Revelation® 4.24 at 450nm. Standard curves were plotted using Microsoft Windows® Excel 2010 spreadsheet software.

3.2.8 Data Entry and Statistical Analysis

All study data (demographic & clinical) that was recorded on clinical study forms or stored in the SALUD software system were transferred manually into an Excel spreadsheet for analysis. In addition, biomarker data (from the analysis of saliva/blood samples in the lab) was exported to the master Excel spreadsheet of the study. For both clinical and protein assay parameters there were paired sets of data for every participant .i.e. before treatment vs. after treatment.

The patient means for all clinical measurements, including plaque and bleeding scores, probing pocket depth and gingival recession were calculated. The probing pocket depth analyses were carried out on deep sites only. i.e. single sites that measured ≥ 5mm at baseline. In order to determine the initial outcome measure to treatment response, we measured the percentage of non-responding deep sites. The non-responding deep sites were defined as sites that did not improve or deteriorated after the non-surgical periodontal treatment. In detail, at site-specific level if the change (pre-operative minus post-operative) in PPD, after treatment, was zero or negative, then the site was assigned as non-responsive.

Thus the percentage of non-responding deep sites =

The number of non-responding deep sites X 100/ Total number of deep sites at baseline.

Postoperatively, participants were classified, based on arbitrarily chosen cut-off (≥ 50%) of the percentage of non-responding deep sites, to either:

Responders: those who had less than 50% of their deep sites which did not respond to the treatment provided. i.e. sites that showed no improvement in the change of their PPD ,

Non-responders: those who had at least 50% of their deep sites which did not respond to the treatment provided.

The dichotomised outcomes “responder” and “non-responder” were used as the primary outcome measure for testing of prognostic markers.

For statistical analyses, Excel, SPSS and Prism® 6 (v 6.04) for Windows MS (GraphPad Software Inc., California, USA) were used. Univariate analyses were carried out to determine:

1. The change in salivary and serum biomarkers in response to treatment, using a paired t-test;
2. The change in salivary and serum biomarkers between responder and non-responder subjects, using un-paired t-tests;
3. The association of pre- and post-operative biomarkers to clinical treatment outcomes, using linear regression;
4. The association of salivary to serum biomarkers, using Pearson correlation;
5. The prognostic predictiveness of pre-operative biomarkers using ROC curves;

In addition, a multivariate model was used to determine clinical and biomarker explanatory variables of treatment outcome. The explanatory variables (potential prognostic factors) tested included mainly salivary biomarkers (MMP-8, HNP1-3 and S100A8) at baseline. The predictive power of these explanatory variables was tested using simple and multiple logistic regressions.

The high correlation among periodontal clinical parameters like probing depth and clinical attachment level causes complexity in order to model these parameters. A simple logistic regression for every explanatory variable was the first step on modelling process. The variable was removed if it did not show statistical significance at a liberal 10% level in the univariate analysis. Then all the significant variables were included in the final multivariate model to find out the significant predictors of treatment response. As multiple regression allows one variable for every 10 participants, we had sufficient power to include up to five parameters.

All statistical analyses were carried out with the supervision of Dr M Andiappan, Lecturer in Statistics, KCL Dental Institute, who also carried out the logistic regression analysis.

3.3 Results

A total of 66 participants were recruited, but only 61 were included in the study (two participants declined treatment and three participants had less than 20 teeth at the time of periodontal assessment .i.e. at the first clinical visit). Whilst the study was progressing a further four participants were excluded from the study because they had started antibiotic therapy after the second visit. The antibiotics had been prescribed by their general medical practitioners to treat systemic and not oral/dental infections. However, the periodontal treatment and reassessment for these four patients was completed as for every other participant in the study. In addition, a further four participants failed to attend the reassessment visit. In total 53 participants completed the study Figure 3-1.

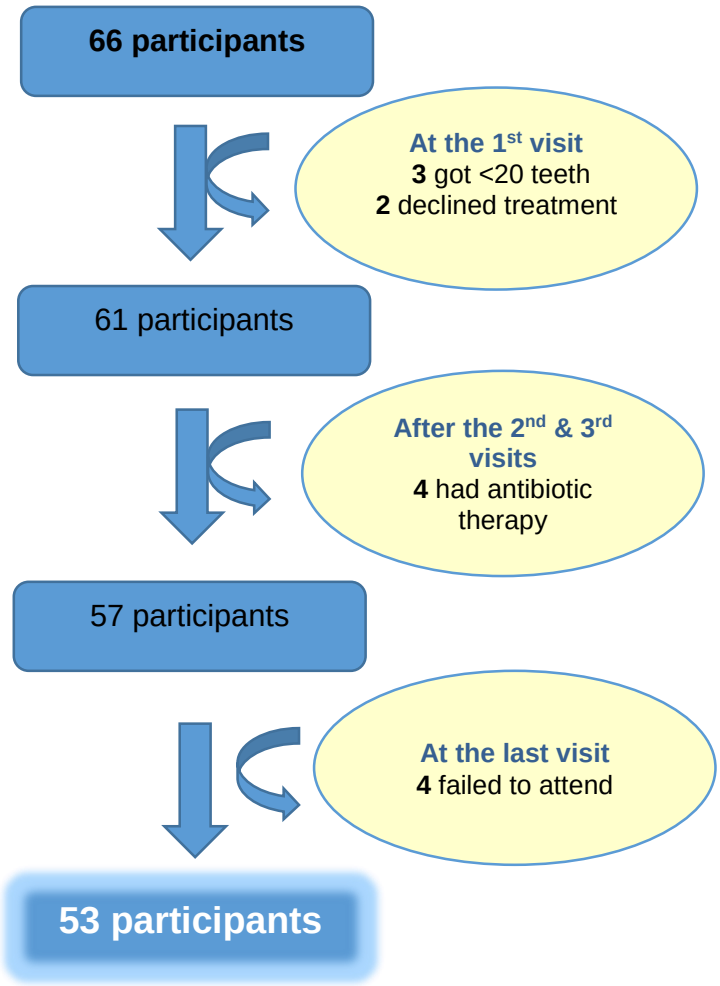

Figure 3-1: Recruitment progress of the study along the four study visits.

From the subjects who completed the study a total of 129 teeth were missing from 42 participants at the baseline assessment (after exclusions of all third molars and first premolars that were extracted for orthodontic reasons). A further two teeth were extracted from two participants after initial assessment and these teeth were excluded from further analysis.

The initial demographic details for the study cohort that completed the study and included in the data analysis, are summarised in Table 3-2 .

Age	Mean 45.49	SD 8.95	Range 23-65
Gender	Male 28	Female 25	
Smoking	Non-smokers 39	Smokers 14	
Diabetes Mellitus	3 (Type II)		
No. of deep sites	Mean 33.57	SD 20.75	Range 4-99
No. of missing teeth	Mean 2.43	SD 2.08	Range 0-9

Table 3-2: Demographic data of the participants who completed the study.

We also calculated the sample size required after the completion of the study to check our initial assumptions in the power calculation, based on comparing the salivary biomarker (MMP-8) levels before and after periodontal treatment using a paired sample t-test. The total number required was 21 participants, with 0.66 the actual effect size and 100% power using post hoc analysis.

3.3.1 Outcome of clinical parameters

Using univariate data analysis of the changes in clinical variables following periodontal treatment, there was a statistically significant reduction in the means of probing pocket depth, number of deep sites (≥ 5mm PPD), plaque and bleeding indices. In addition, there was a statistically significant increase in the gingival recession Table 3-3.

Clinical Variable	Before treatment (Mean ± SD)	After treatment (Mean ± SD)	p value
Probing Pocket Depth	5.92mm ± 0.47mm	4.74mm ± 0.76mm	**<0.0001***
Number of Deep Sites	33.57 ± 20.75	18.51 ± 13.87	**<0.0001***
Plaque Index	0.46 ± 0.20	0.37 ± 0.18	**0.0003***
Bleeding Index	0.32 ± 0.20	0.21 ± 0.16	**<0.0001***
Gingival Recession	2.65mm ± 0.99mm	3mm ± 0.91mm	**<0.0001***

***** Statistically significant ($p<0.05$).

Table 3-3: Change in clinical variables after periodontal treatment using 2- tailed paired t-test.

3.3.1.1 Individual Patient Responses

As described earlier, we used the "percentage of non-responding deep sites" as our primary outcome measure, where participants were classified accordingly to responders and non-responders.

In order to dichotomize the study participants according to their response to the provided treatment, we used a cut-off point of the percentage of non-responding deep sites (≥50%). Therefore, all participants that showed an improvement of at least 50% or more of their deep sites after treatment, were classified as responders and vice versa.

Applying this cut-off (≥50%) to the results of this study, out of 53 participants there were 40 responders and 13 non-responders .i.e. only 24.5% of the chronic periodontitis cases did not respond to the non-surgical periodontal treatment Figure 3-2.

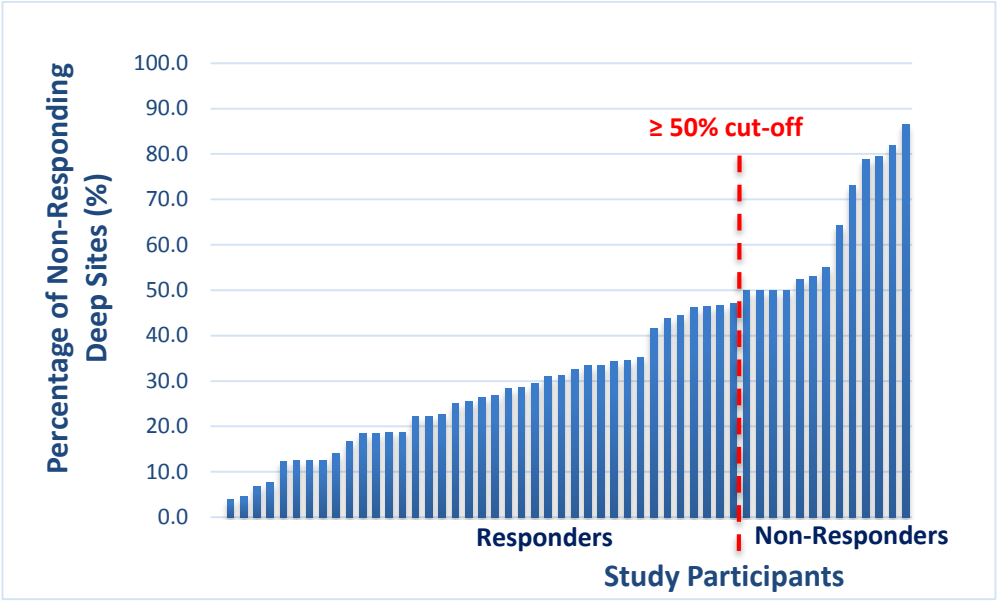

Figure 3-2: Clustered column chart shows the dichotomised participants according to the cut-off (≥50%) of the percentage of non-responding deep sites over periodontal treatment.

In addition, in terms of the change in the mean of probing pocket depth after non-surgical periodontal treatment, most of the cases showed positive change, and only three showed overall deterioration after treatment Figure 3-3.

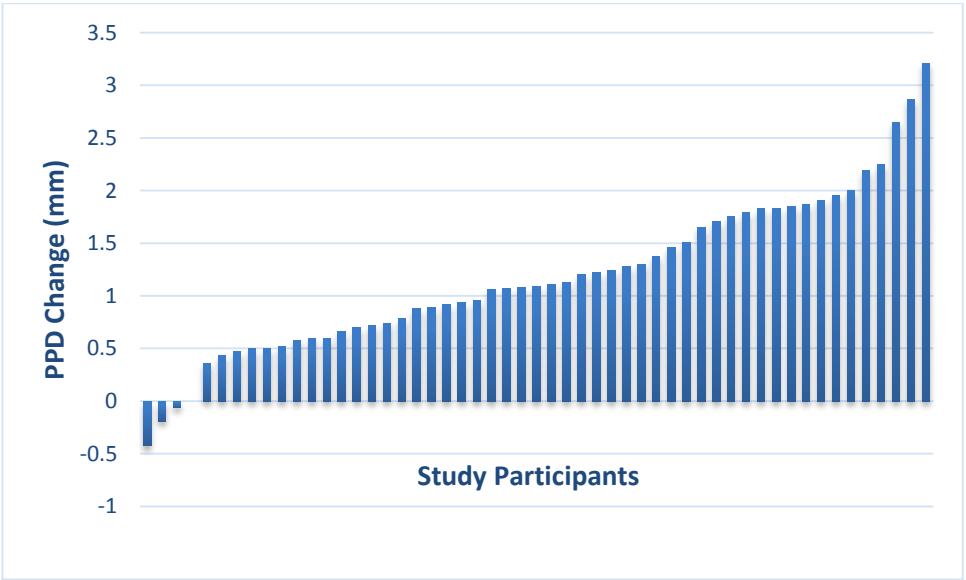

Figure 3-3: Clustered column chart shows the change in the mean of probing pocket depth (PPD) over periodontal treatment.

There was a strong correlation between both of these clinical outcomes (percentage of non-responding deep sites and PPD change) (r = - 0.9, p<0.0001) Figure 3-4.

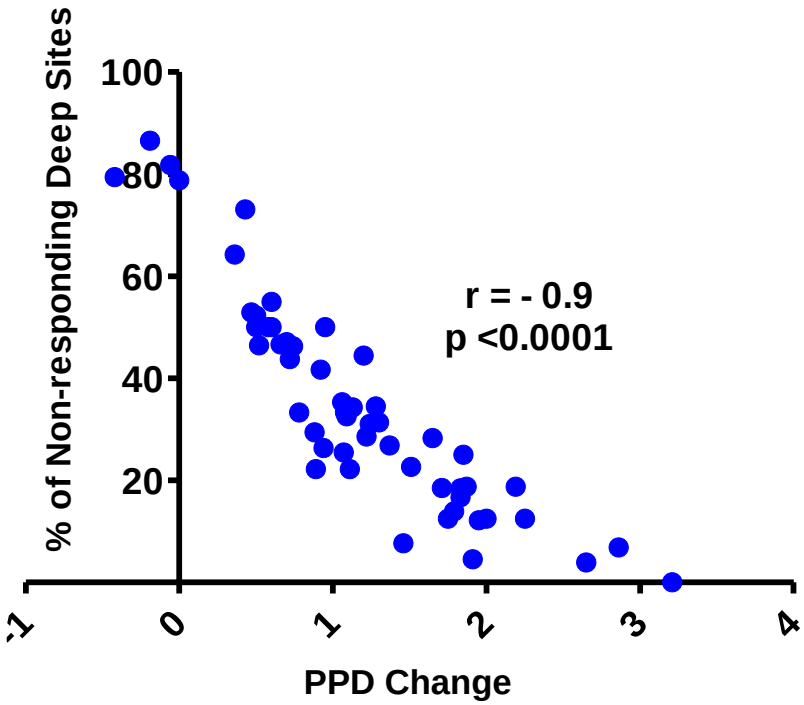

Figure 3-4: Scatter plot graph showing the significant correlation (p<0.0001) between the percentage of non-responding deep sites and change in PPD.

3.3.1.2 Clinical Parameters in Responders vs. Non-responders

The demographic data of the participants was not significantly different between responders and non-responders Table 3-4. However, there were statistically significant reductions in the change of both probing pocket depth and number of deep sites between responders and non-responders. On the other hand, there was no statistical significant difference between the two groups in terms of change in plaque & bleeding indices and gingival recession. Also, both the number of deep sites and probing pocket depth at baseline were not statistically significant between the two groups Table 3-5.

Variable	Responders	Non-responders	P Value
Total Number	40	13	
Age	Mean 45.2	Mean 46.4	NS**
Gender	Female 19, Male 21	Female 6, Male 7	NS*
Smoking	Non-smokers 29, Smokers 11	Non-smokers 10, Smokers 3	NS *
Diabetes	3	0	

***P value by Fishers Exact Test, **P value by unpaired t-test.**

Table 3-4: Demographic data of the responders versus non-responders.

Clinical Variable	Responders (Mean ± SD)	Non-responders (Mean ± SD)	p value
Probing Pocket Depth (Change)	1.45mm ± 0.62mm	0.33mm ± 0.38mm	**<0.0001 ***
Probing Pocket Depth (Baseline)	5.87mm ± 0.48mm	6.05mm ± 0.41mm	0.2
Number of Deep Sites (Change)	17.95 ± 13.39	6.15 ± 4.14	**<0.0031 ***
Number of Deep Sites (Baseline)	34.68 ± 22.70	30.15 ± 13.20	0.5
Plaque Index (Baseline)	0.46 ± 0.19	0.46 ± 0.22	1
Plaque Index (Change)	0.09 ± 0.16	0.08 ± 0.17	0.9
Bleeding Index (Baseline)	0.32 ± 0.18	0.31 ± 0.21	0.8
Bleeding Index (Change)	0.12 ± 0.18	0.06 ± 0.10	0.2
Gingival Recession (Change)	-0.35mm ± 0.50mm	-0.31mm ± 0.65mm	0.8

***** Statistically significant ($p<0.05$).

Table 3-5: Comparison of clinical variables in responders versus non-responders by 2-tailed unpaired t-test.

3.3.2 Clinical Data Intra-examiner Reproducibility

A total of eight participants were randomly selected in order to re-measure both probing pocket depth (PPD) and recession (Rec) of half-mouth (two quadrants), at the same visit (either at baseline or reassessment visits). The side selection was random; four participants were selected for the right side and four for the left side. All the readings were carried out by the same clinical researcher (BK). A total of 560 sites were included in the reproducibility study.

3.3.2.1 Using the Means of Probing Pocket Depth & Recession

The correlations between the first and second measurements for (PPD) and (Rec) are 0.85 and 0.89 respectively. Both the correlations are statistically significant ($p<0.0001$) indicating the two measurements are linearly related but does not necessarily mean that there is a good agreement between the two measurements. Hence, a Bland-Altman graph was drawn by taking the mean of the two measurements (first and second measurement) and the difference between the two measurements. The graphs are shown in Figure 3-5 for PPD and Figure 3-6 for REC. From the graph it is clear that there is no systematic difference (fixed bias) and all the data points lie below mean $\pm$ 2 SD. Further analysis of one sample t-test to test whether the mean difference is different showed that the mean difference is not significantly different ($p=0.40$ for PPD and $p=0.14$ for REC) from 0. This indicates that there is no fixed bias in the measurement.

Linear regression between mean and difference of the two measurements showed that there is no proportional bias between the two measurements (the slope for both PPD and REC were not significant). Hence it can be concluded that both the measurements agree with reasonable precision.

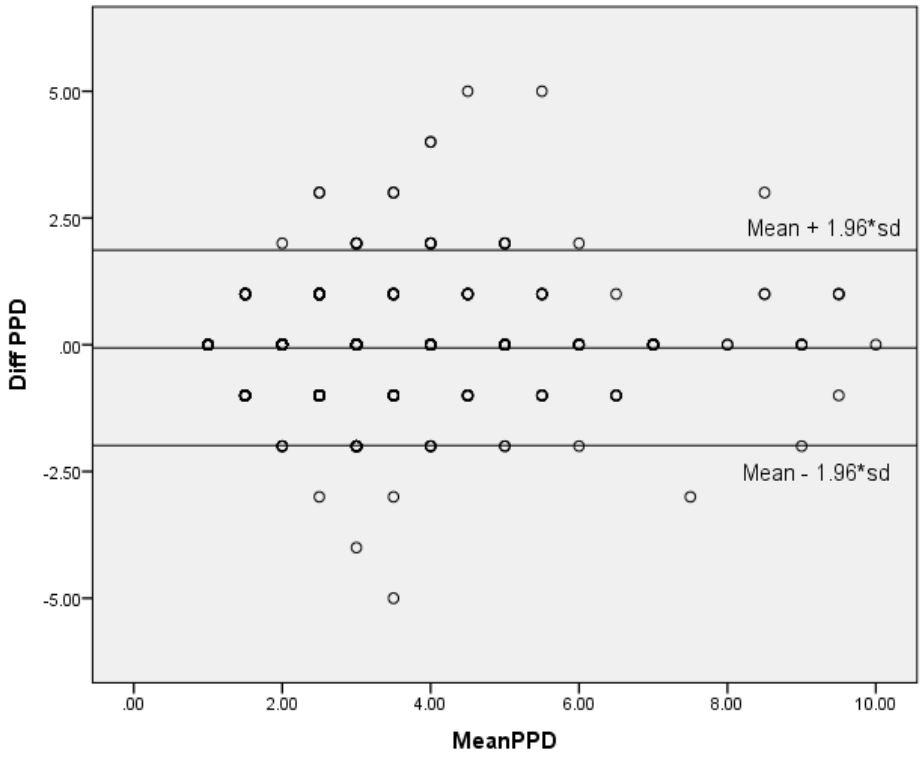

Figure 3-5: Bland Altman graph for PPD.

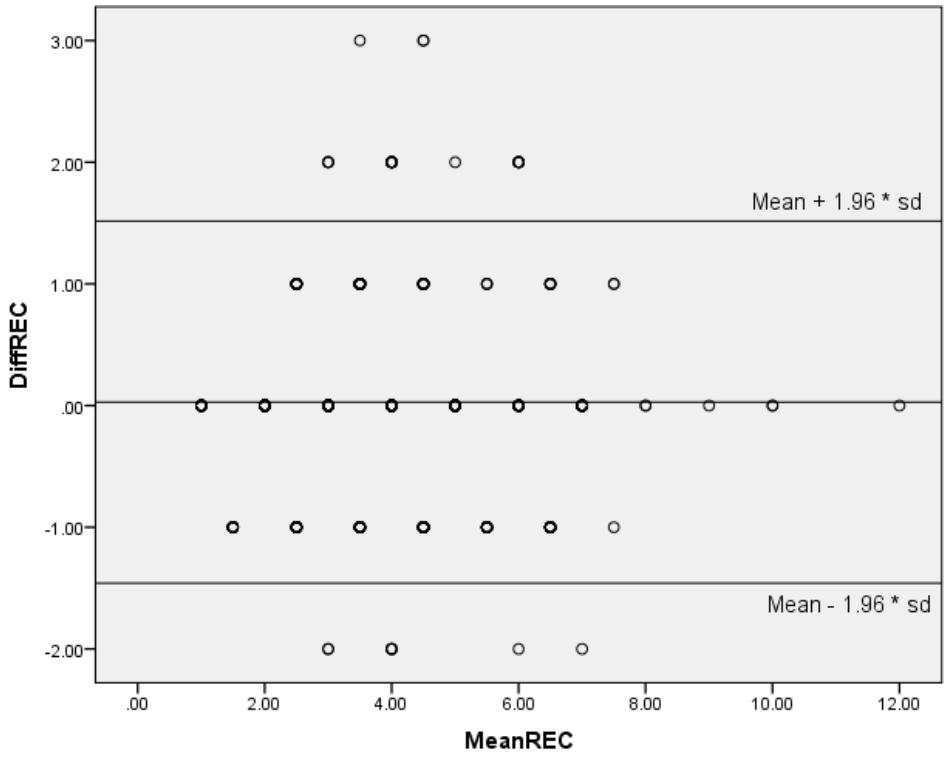

Figure 3-6: Bland Altman graph for REC.

3.3.2.2 Using the Actual Individual Readings for Probing Pocket Depth & Recession

As a difference of more than 2 mm is considered to be a significant difference, the differences in the PPD and REC values between the two ratings were as below Table 3-6 and

Table 3-7.

Difference	No. of sites	% of sites
0	331	59.1
1	171	30.5
2	44	7.9
>2	14	2.5
Total	560	100.0

Table 3-6: Shows the probing pocket depth (PPD) difference between the two readings.

Difference	No. of sites	% of sites
0	301	62.3
1	155	32.1
2	24	5.0
>2	3	0.5
Total	483	100.0

Table 3-7: Shows the recession (REC) difference between the two readings.

From Table 3-6 and Table 3-7, it is clear that the two measurements for PPD and REC are ≤ 2 in 97.5% and 99.5% of sites respectively. Hence there is a good intra-examiner agreement. The mean difference for PPD and REC was 0.55 and 0.44 respectively, which is much lower than the threshold of 2 and hence we can conclude that both ratings are agreeing.

3.3.3 Biochemical Parameters

3.3.3.1 Salivary Biomarkers

The levels of MMP-8 and S100A8 reduced dramatically after the non-surgical periodontal treatment Table 3-8, Figure 3-7 and Figure 3-8. Despite the apparent reduction in HNP1-3 concentrations after treatment, this reduction did not reach statistical significance Table 3-8 and Figure 3-9.

Salivary Biomarker	Baseline (Mean ± SD)	Review (Mean ± SD)	P value
MMP-8 (ng/ml)	355.4 ± 319.9	216.6 ± 217.2	**<0.0001***
HNP1-3 (µg/ml)	4.6 ± 6.6	3.4 ± 4.4	0.3
S100A8 (ng/ml)	1182 ± 1095	693.9 ± 719.6	**0.0007***

Table 3-8: Changes in salivary biomarkers following periodontal treatment using paired t-test.

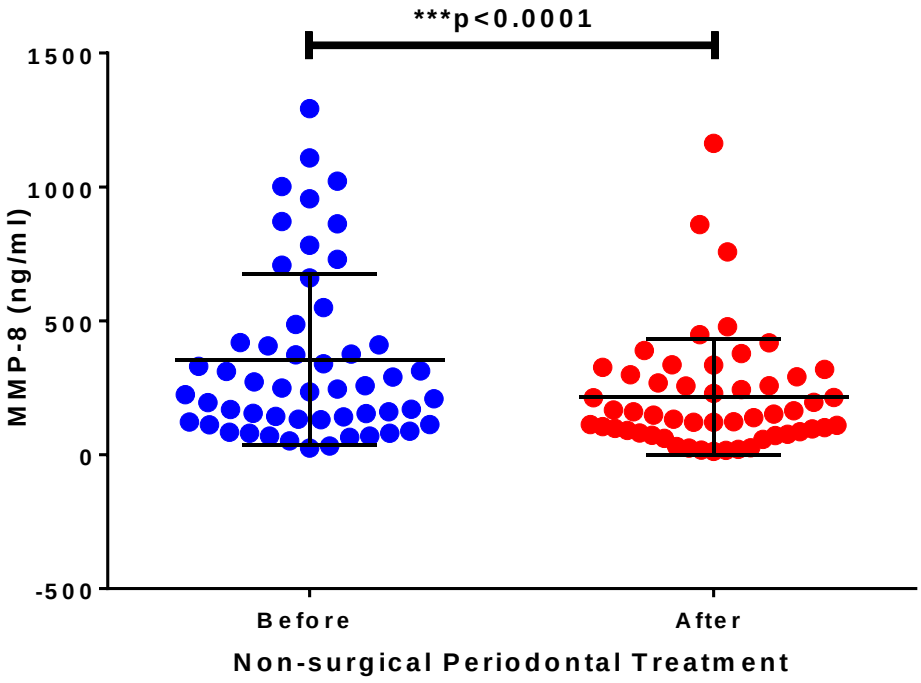

Figure 3-7: Vertical Scatter plot chart showing the significant reduction in MMP-8 levels over non-surgical periodontal treatment.

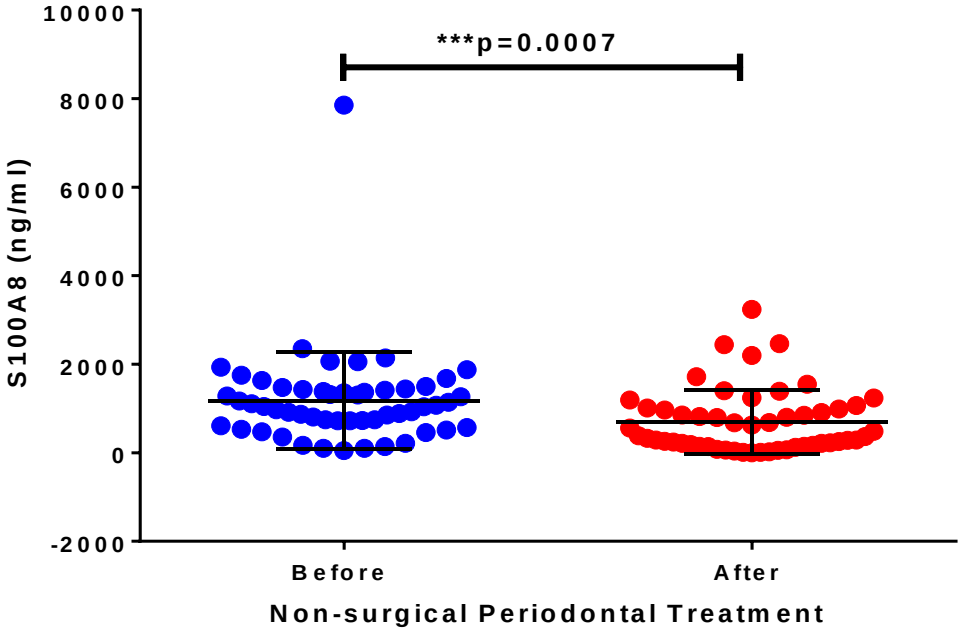

Figure 3-8: Vertical scatter plot chart showing the significant change in S100A8 levels over non-surgical periodontal treatment.

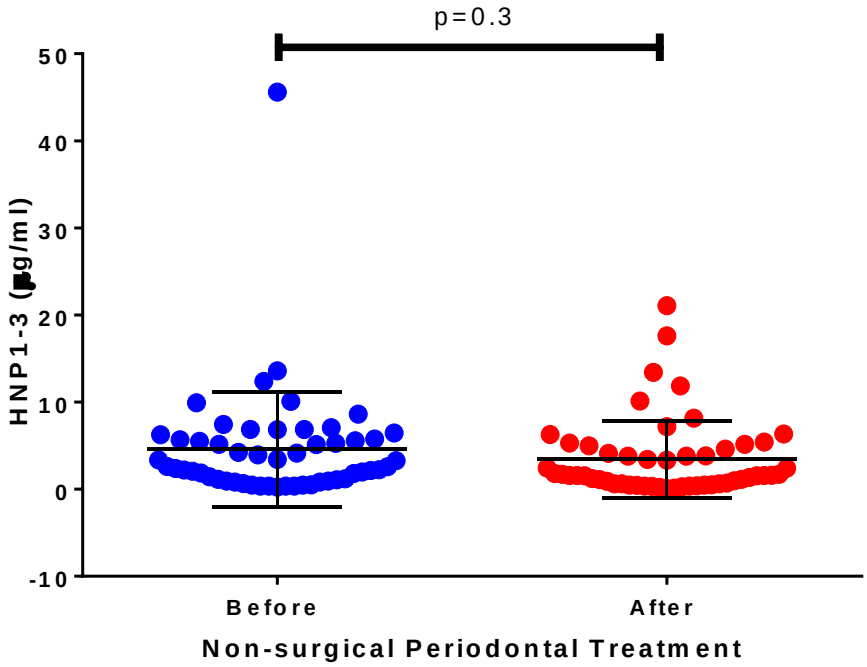

Figure 3-9: Vertical scatter plot chart showing the non-significant change in HNP1-3 levels over non-surgical periodontal treatment.

3.3.3.2 Serum Markers

Neither CRP nor IL-6 concentration showed any statistically significant reduction postoperatively Table 3-9, Figure 3-10 and Figure 3-11.

Serum Marker	Baseline (Mean ± SD)	Review (Mean ± SD)	P value
CRP (mg/L)	4.7 ± 6	4.3 ± 6.5	0.6
IL-6 (pg/ml)	1.2 ± 1	1.1 ± 0.9	0.7

Table 3-9: Changes in serum biomarkers after periodontal treatment using paired t-test.

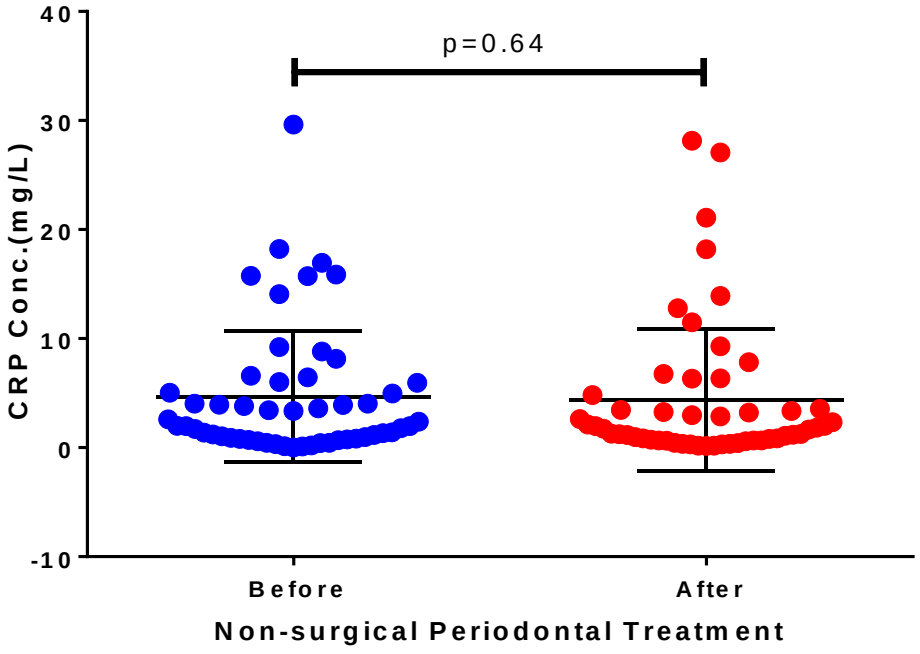

Figure 3-10: Vertical scatter plot chart showing a non-significant change in CRP levels over non-surgical periodontal treatment.

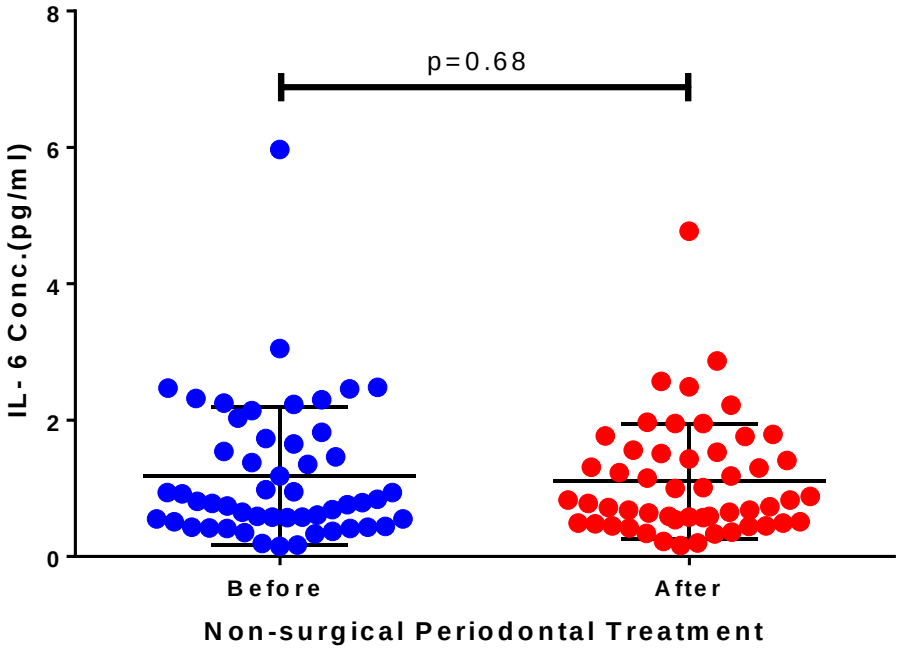

Figure 3-11: Vertical scatter plot chart showing a non-significant change in IL- 6 levels over non-surgical periodontal treatment.

A summary of the univariate analyses of clinical and biochemical treatment outcomes are shown in Table 3-10:

Variable	Before treatment (Mean ± SD)	After treatment (Mean ± SD)	p value
Probing Pocket Depth (mm)	5.92 ± 0.47	4.74 ± 0.76	**<0.0001***
Number of Deep Sites	33.57 ± 20.75	18.51 ± 13.87	**<0.0001***
Plaque Index	0.46 ± 0.20	0.37 ± 0.18	**0.0003***
Bleeding Index	0.32 ± 0.20	0.21 ± 0.16	**<0.0001***
Gingival Recession (mm)	2.65 ± 0.99	3 ± 0.91	**<0.0001***
MMP-8 (ng/ml)	355.4 ± 319.9	216.6 ± 217.2	**<0.0001***
HNP1-3 (µg/ml)	4.6 ± 6.6	3.4 ± 4.4	0.3
S100A8 (ng/ml)	1182 ± 1095	693.9 ± 719.6	**0.0007***
CRP (mg/L)	4.7 ± 6	4.3 ± 6.5	0.6
IL-6 (pg/ml)	1.2 ± 1	1.1 ± 0.9	0.7

* Statistically significant (p<0.05).

Table 3-10: Summary of clinical and biochemical changes after periodontal treatment using two-tailed paired t-test.

3.3.3.3 Biochemical Outcomes in Responders vs. Non-responders

Before periodontal treatment, only salivary MMP-8 showed a statistically significant difference between responders and non-responders at baseline level, with responders having a significantly higher baseline concentration of MMP-8 compared with non-responders Table 3-11 and Figure 3-12 . After treatment, none of the tested markers showed any statistical difference between the two groups at outcome level Table 3-11.

Biochemical Variable	Responders (Mean ± SD)	Non-responders (Mean ± SD)	p value
MMP-8 Baseline (ng/ml)	419.3 ± 343.1	158.8 ± 73.3	**0.009***
MMP-8 Outcome (ng/ml)	238.2 ± 235.2	150.1 ± 135.5	0.2
HNP1-3 Baseline (µg/ml)	5.0 ± 7.4	3.2 ± 3.0	0.4
HNP1-3 Outcome (µg/ml)	3.5 ± 4.4	3.2 ± 4.5	0.9
S100A8 Baseline (ng/ml)	1305 ± 1211	802.2 ± 470.1	0.2
S100A8 Outcome (ng/ml)	742 ± 726.8	546 ± 703.9	0.4
CRP Baseline (mg/L)	5.4 ± 6.3	2.6 ± 4.4	0.1
CRP Outcome (mg/L)	4.9 ± 7.1	2.6 ± 3.7	0.3
IL-6 Baseline (pg/ml)	1.3 ± 1.1	0.8 ± 0.5	0.1
IL-6 Outcome (pg/ml)	1.2 ± 0.9	0.9 ± 0.6	0.3

* Statistically significant ($p<0.05$).

Table 3-11: Analysis of the biochemical variables before and after periodontal treatment in responders versus non-responders using unpaired t-test (two tailed p value).

In terms of change in both salivary (MMP-8, HNP1-3 and S100A8) and systemic markers (CRP and IL-6), only MMP-8 showed a statistically significant difference between responders and non-responders Table 3-12 and Figure 3-13. The levels of HNP1-3, S100A8, CRP and IL-6 did not show any significant difference between the two groups after periodontal treatment Table 3-11.

Biochemical Variable	Responders (Mean ± SD)	Non-responders (Mean ± SD)	p value
MMP-8 Change (ng/ml)	181.1 ± 240.3	8.7 ± 106.1	**0.02***
HNP1-3 Change (µg/ml)	1.5 ± 5.2	-0.1 ± 3.5	0.3
S100A8 Change (ng/ml)	563 ± 1055	256.3 ± 726	0.3
CRP Change (mg/L)	0.4 ± 5.8	-0.004 ± 2.3	0.8
IL-6 Change (pg/ml)	0.1 ± 1	-0.1 ± 0.6	0.5

* Statistically significant ($p<0.05$).

Table 3-12: Changes in biochemical variables in responders versus non-responders using unpaired t-test (two tailed p value).

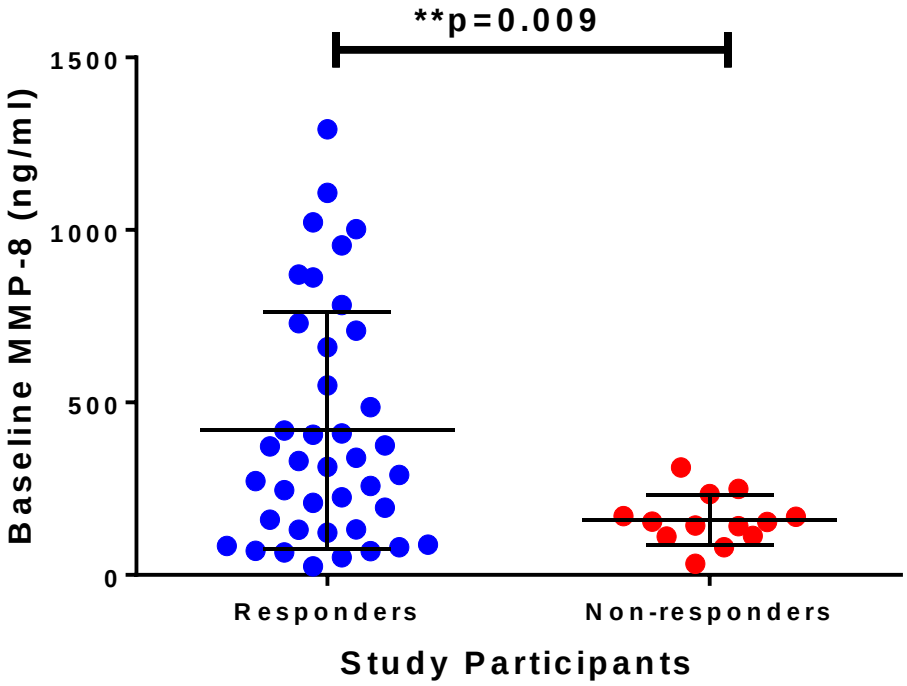

Figure 3-12: Column scattered plot graph showing the statistically significant difference in the MMP-8 baseline levels between responders and non-responders before periodontal treatment.

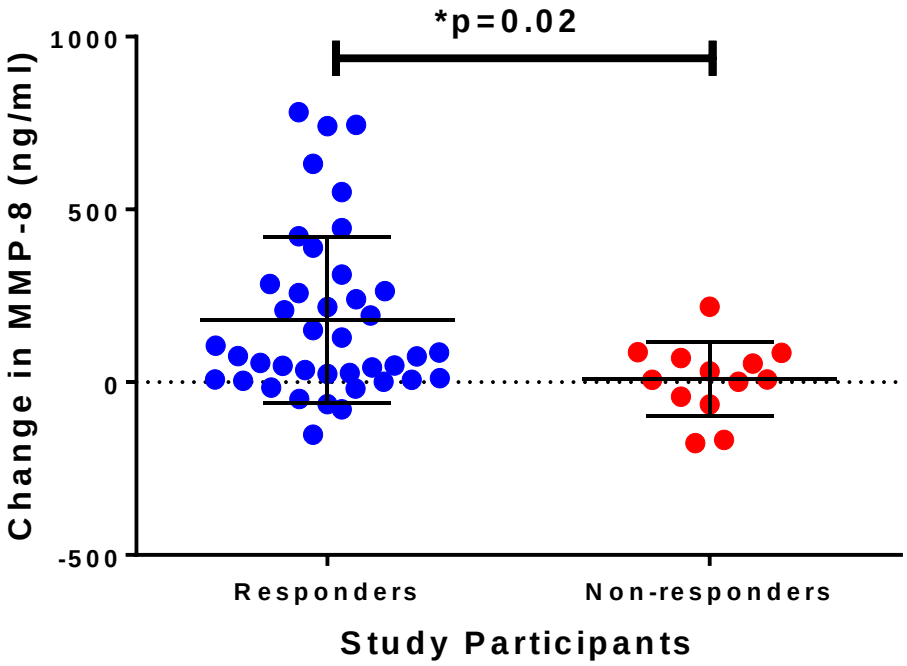

Figure 3-13: Column scattered plot graph showing the statistically significant difference in the change of MMP-8 levels between responders and non-responders after periodontal treatment.

3.3.4 Clinical & Biochemical Outcomes in Smokers vs. Non-smokers

As showed earlier, out of 53 participants in this study there were 14 smokers and 39 non-smokers. The two-tailed unpaired t-test showed no significant difference in terms of all clinical and biochemical parameters at baseline level (before treatment) Table 3-13 and Table 3-14.

In terms of changes of these parameters after periodontal treatment, the only variable that showed significant difference between the two groups is the change in plaque index (PI) in which the PI change was higher in non-smokers than smokers Table 3-13 and Figure 3-14.

Clinical Variable	Non-smokers (Mean ± SD)	Smokers (Mean ± SD)	p value
Probing Pocket Depth (Baseline)	5.90mm ± 0.47mm	5.96mm ± 0.47mm	0.7
Probing Pocket Depth (Change)	1.18mm ± 0.77mm	1.19mm ± 0.70mm	1
Number of Deep Sites (Baseline)	34.05 ± 22.63	32.21 ± 14.91	0.8
Number of Deep Sites (Change)	15.31 ± 13.82	14.36 ± 9.99	0.8
Plaque Index (Baseline)	0.48± 0.20	0.41 ± 0.18	0.3
Plaque Index (Change)	0. 11± 0.16	0.002 ± 0.13	**0.02***
Bleeding Index (Baseline)	0.32 ± 0.21	0.31 ± 0.10	0.7
Bleeding Index (Change)	0.10 ± 0.18	0.12 ± 0.10	0.8
Gingival Recession (Baseline)	2.78mm ± 1.05mm	2.31mm ± 0.76mm	0.1
Gingival Recession (Change)	-0.34mm ± 0.60mm	-0.35mm ± 0.33mm	0.9

***** Statistically significant ($p<0.05$).

Table 3-13: Comparison of baseline and changes in clinical variables in smokers versus non-smokers following periodontal treatment, using unpaired t-test (two tailed p value).

Biochemical Variable	Non-smokers (Mean ± SD)	Smokers (Mean ± SD)	p value
MMP-8 Baseline (ng/ml)	354.7 ± 324.2	357.5 ± 319.7	1
MMP-8 Change (ng/ml)	149.7 ± 229.5	108.7 ± 225.3	0.6
HNP1-3 Baseline (µg/ml)	5.4 ± 7.4	3.3 ± 2.6	0.1
HNP1-3 Change (µg/ml)	1.1 ± 5.5	1.1 ± 2.1	1
S100A8 Baseline (ng/ml)	1273 ± 1252	928.7 ± 348.2	0.3
S100A8 Change (ng/ml)	492.9 ± 1119	473.4 ± 485.7	0.9
CRP Baseline (mg/L)	4.6 ± 6.1	4.9 ± 6	0.8
CRP Change (mg/L)	0.2 ± 5.8	0.7 ± 2.3	0.7
IL-6 Baseline (pg/ml)	1.2 ± 1	1.2 ± 1	0.8
IL-6 Change (pg/ml)	0.1 ± 0.9	0 ± 0.9	0.6

* Statistically significant ($p<0.05$).

Table 3-14: Comparison of baseline and changes in biochemical variables before and after periodontal treatment in smokers versus non-smokers, using unpaired t-test (two tailed p value).

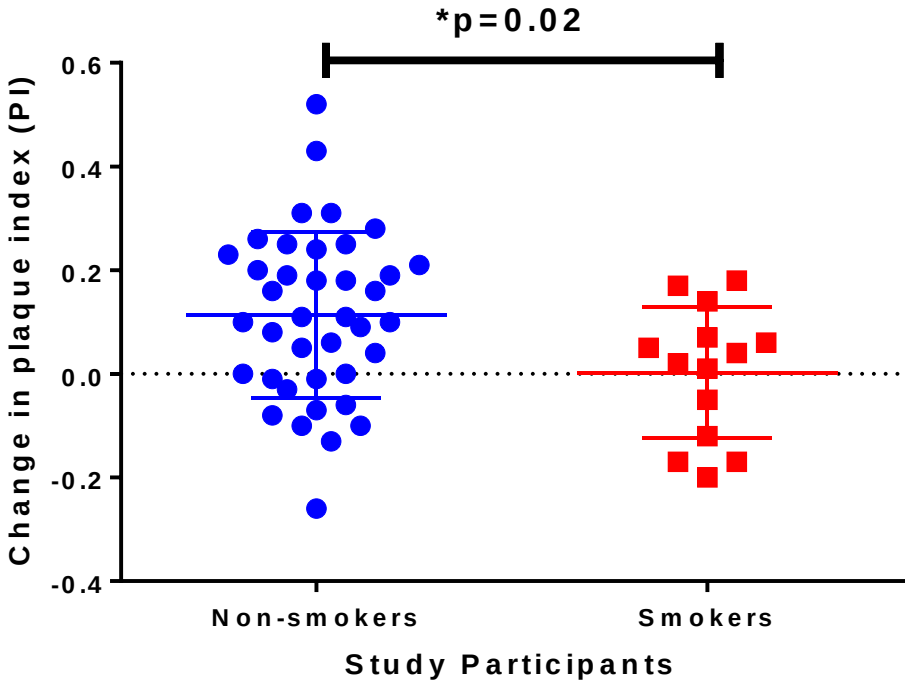

Figure 3-14: Column scattered plot graph showing the statistical significance difference in the change of plaque index between smokers and non-smokers after periodontal treatment.

3.3.5 Associations

3.3.5.1 Association of Change in Biomarkers with Treatment Outcome

	MMP-8 Change	HNP1-3 Change	S100A8 Change	CRP Change	IL-6 Change
Outcome Indicator (PPD Change)	r^2=0.1 ***p=0.02**	r^2=0.01 p=0.4	r^2=0.1 p=0.05	r^2=0.02 p=0.4	r^2=0.01 p=0.5

* Statistically significant (p<0.05).

Table 3-15: Linear regression models the relationship between the change in biomarkers, after periodontal treatment, and treatment outcome.

Linear regressions were used to model the change of biomarkers and treatment outcome between baseline and post-treatment, and the effect of biomarkers and demographic variables on treatment outcome. We used the change in the probing pocket depth (PPD) as our treatment outcome indicator (as we showed earlier in the results that the probing pocket depths were significantly reduced after treatment ($p<0.0001$)).

After periodontal treatment, the only biomarker that showed a significant association between the change in concentration and the treatment outcome (change in probing pocket depth) was MMP-8 Table 3-15 and Figure 3-15. However, this relationship was weak. The change of salivary HNP1-3 & S100A8, and serum CRP & IL-6, after treatment, did not show any statistical association with treatment outcome Table 3-15.

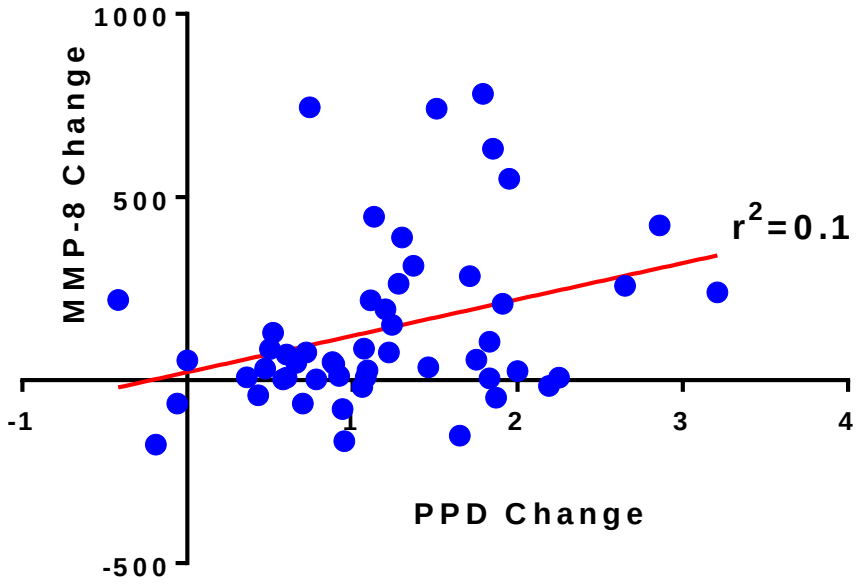

Figure 3-15: Scatter plot graph shows the significant association (p=0.02) of treatment outcome to the change in salivary MMP-8, over periodontal treatment.

3.3.5.2 Association of Biomarker Baseline Levels with Treatment Outcome

	MMP-8 baseline	HNP1-3 baseline	S100A8 baseline	CRP baseline	IL-6 baseline
Outcome Indicator (PPD Change)	r^2=0.1 ***p=0.03**	r^2=0.02 p=0.3	r^2=0.1 ***p=0.02**	r^2=0.02 p=0.3	r^2=0.006 p=0.6

* Statistically significant (p<0.05).

Table 3-16: Linear regression models the relationship between the baseline levels of biomarkers and treatment outcome.

To investigate the prognostic utility of these biomarkers using linear regressions again in order to model the association of the baseline levels (before treatment) of these biomarkers to the treatment outcome.

The baseline levels of salivary biomarkers MMP-8 and S100A8 showed a statistically significant association with the treatment outcome Table 3-16, Figure 3-16 and Figure 3-17. However the relationship was weak. The baseline levels of salivary HNP1-3 and serum CRP & IL-6, did not show any statistical association with treatment outcome Table 3-16.

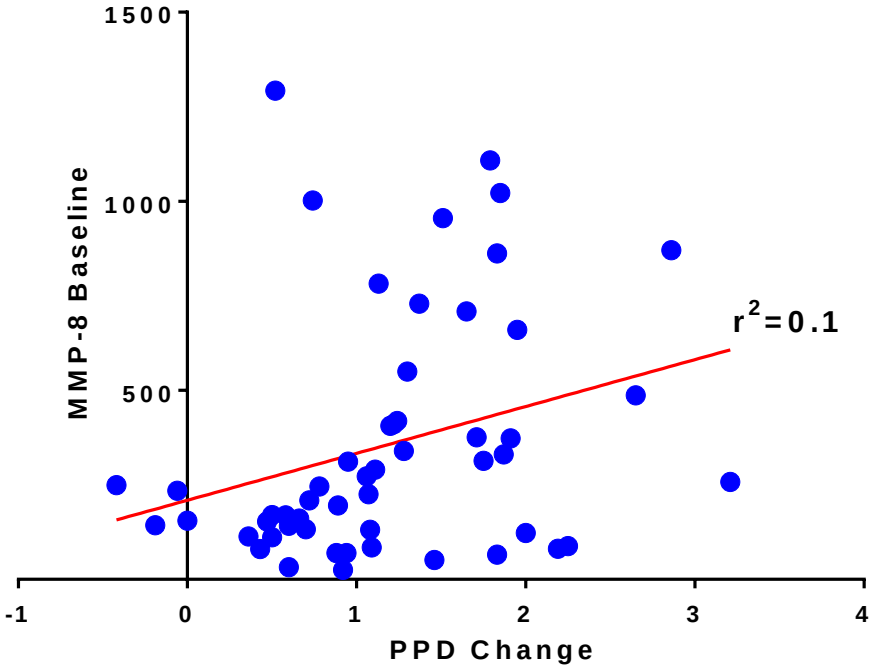

Figure 3-16: Scatter plot graph shows the significant association (p=0.03) of treatment outcome to the baseline levels of salivary MMP-8.

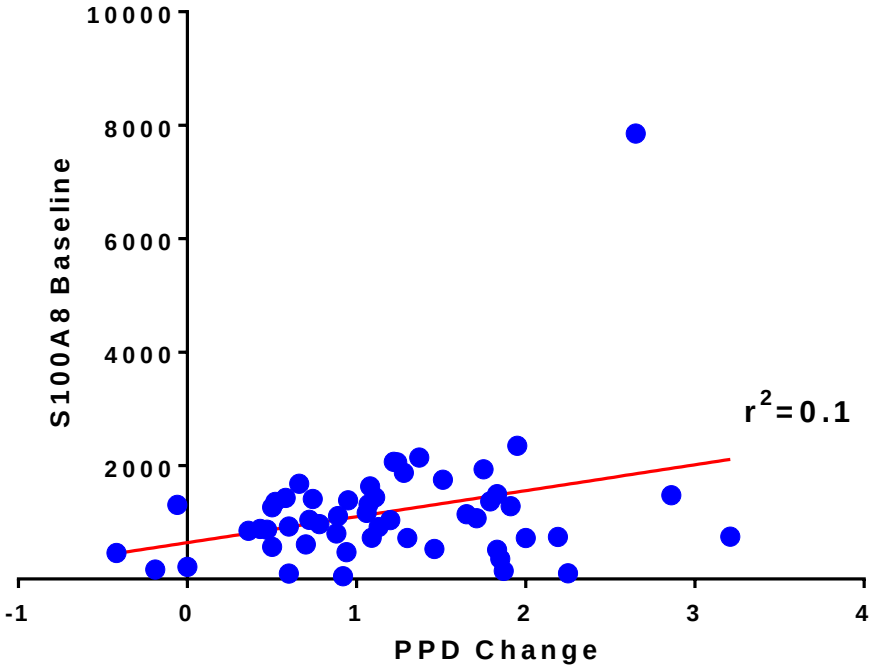

Figure 3-17: Scatter plot graph shows the significant association (p=0.02) of treatment outcome to the baseline levels of salivary S100A8.

3.3.6 Prognostic Utility of Salivary/ Systemic Markers

In order to determine a cut-off point for the prognostic utilities of these markers we carried out ROC curve analysis of the markers' baseline levels of responders versus non-responders. From the results of previous analysis MMP-8 was the only biomarker that showed a statistically significant difference between the two groups at baseline level (before treatment) (*p=0.009).

ROC curve analysis of the MMP-8 baseline levels in responders versus non-responders showed that MMP-8 baseline concentration at cut-off point of (<182.8 ng/ml) can predict the responsiveness to periodontal treatment with 77% sensitivity and 70% specificity Figure 3-18.

Interestingly, if we increase the baseline MMP-8 cut-off to <253.4 ng/ml in order to achieve high sensitivity of 92.3%, the specificity remained reasonably high at 60%. On the other hand, dropping the cut-off to <41.91 ng/ml in order to achieve high specificity of 97.5%, the sensitivity dropped dramatically to 7.7%.

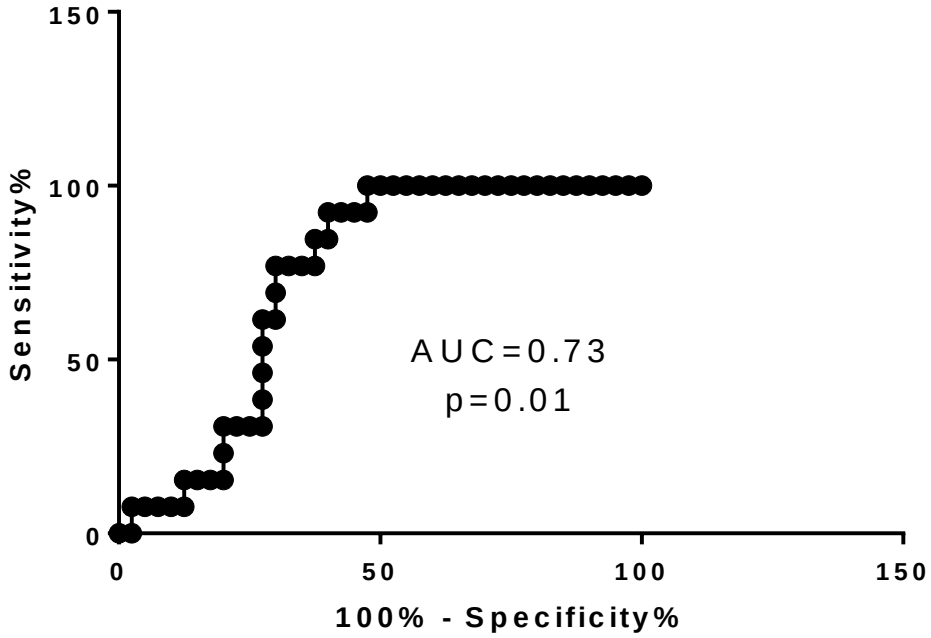

Figure 3-18: ROC curve construct for salivary MMP-8 baseline levels with significant ability to distinguish between responders and non-responders.

3.3.7 Salivary Biomarkers vs. Systemic Markers (Correlations)

In order to investigate the relationship between local (salivary) biomarkers and systemic (serum) markers, we ran a Pearson correlation statistical analysis to check the relationship of both baseline levels and change (baseline level minus outcome level) over treatment of these local/systemic markers to each other.

3.3.7.1 Correlation between the baseline levels of salivary/systemic markers

	MMP-8 Baseline	HNP1-3 Baseline	S100A8 Baseline	CRP Baseline	IL-6 Baseline
MMP-8 Baseline		r=0.6 ***p<0.0001**	r=0.3 p=0.05	r=0.2 p=0.2	r=0.2 p=0.09
HNP1-3 Baseline	r=0.6 ***p<0.0001**		r=0.1 p=0.6	r= - 0.03 p=0.8	r=0.2 p=0.2
S100A8 Baseline	r=0.3 p=0.05	r=0.1 p=0.6		r=0.1 p=0.4	r= - 0.05 p=0.7
CRP Baseline	r=0.2 p=0.2	r= - 0.03 p=0.8	r=0.1 p=0.4		r=0.5 ***p=0.0003**
IL-6 Baseline	r=0.2 p=0.09	r=0.2 p=0.2	r= - 0.05 p=0.7	r=0.5 ***p=0.0003**	

* Statistically significant (p<0.05)

Table 3-17: Pearson correlation table demonstrates the relationship between the salivary and systemic markers at baseline level.

As we can see from the results of the above Table 3-17 there was a moderate positive correlation between baseline concentrations of salivary MMP-8 & HNP1-3 Figure 3-19 and between baseline measurements of CRP & IL-6 Figure 3-20. No other significant correlations were seen (Pearson correlation, two-tailed p value).

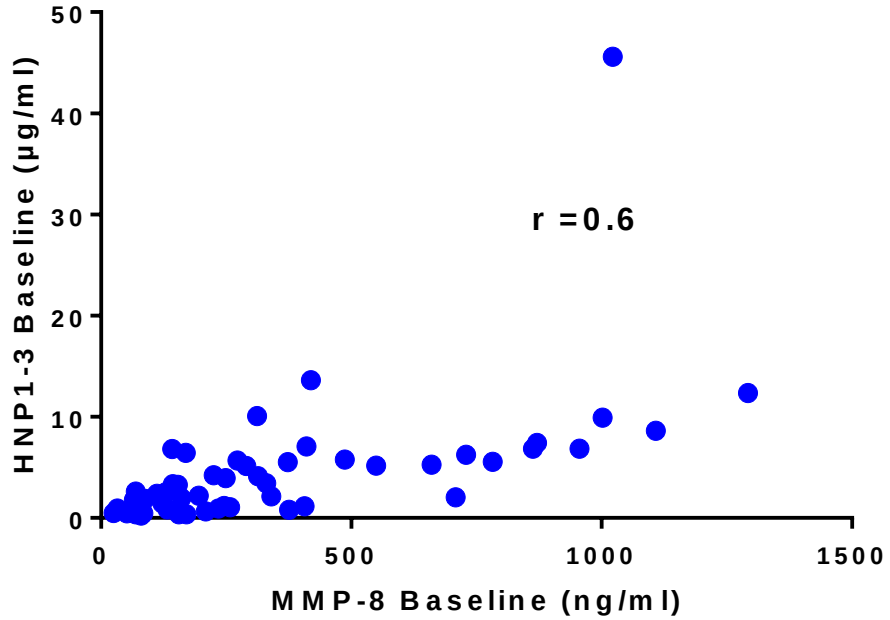

Figure 3-19: Scatter plot graph showing the significant correlation (p<0.0001) between the baseline levels of salivary MMP-8 & HNP1-3.

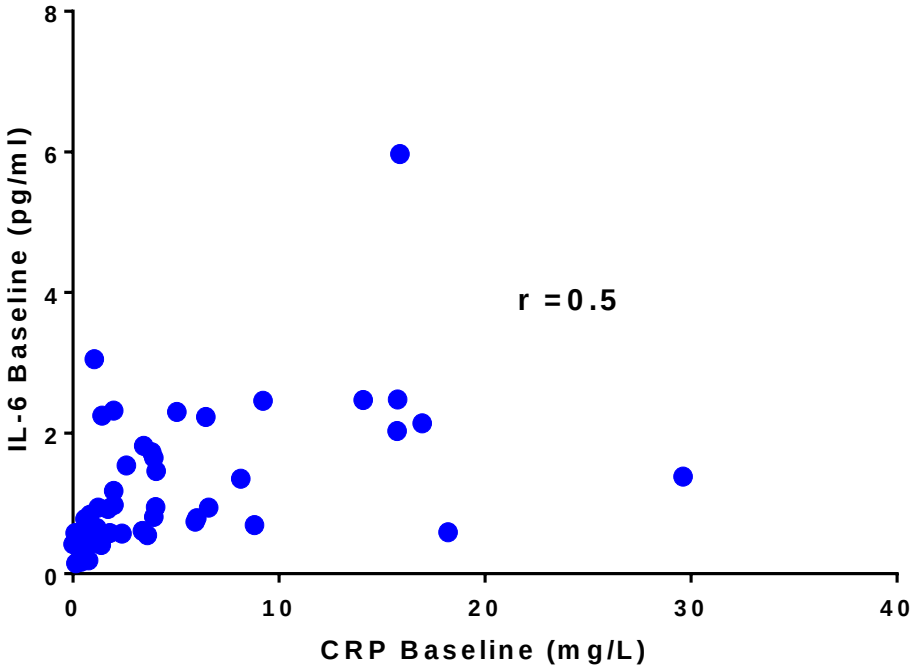

Figure 3-20: Scatter plot graph showing the significant correlation (p=0.0003) between the baseline levels of systemic CRP & IL-6.

The baseline level correlations of serum CRP to salivary MMP-8 and S100A8 were non-significant (Pearson correlation, two-tailed p value) Table 3-17, Figure 3-21 and Figure 3-22.

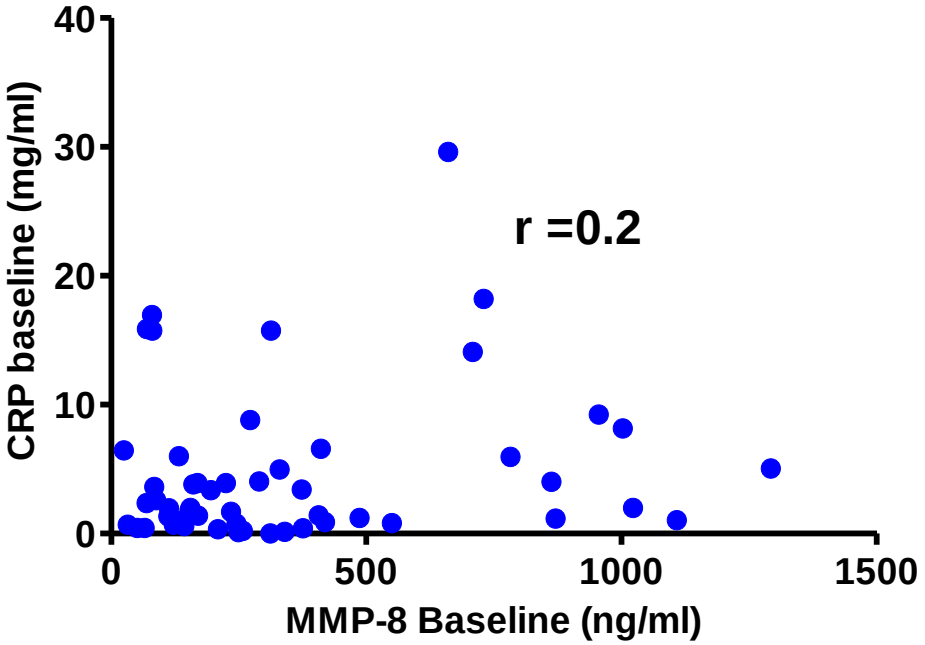

Figure 3-21: Scatter plot graph showing the non-significant correlation (p=0.2) between the baseline levels of systemic CRP & salivary MMP-8.

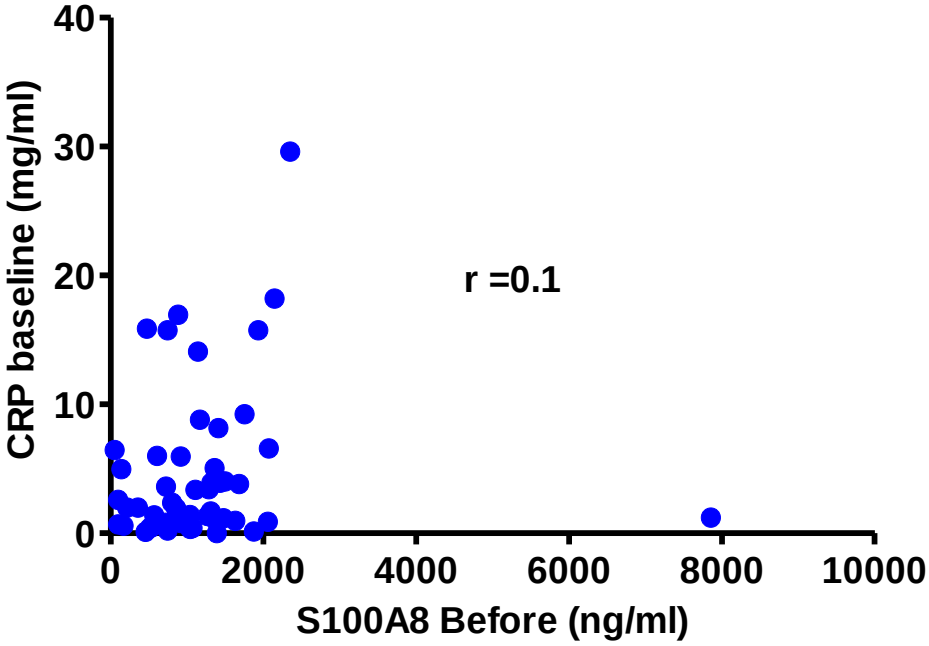

Figure 3-22: Scatter plot graph showing the lack of correlation (p=0.4) between the baseline levels of systemic CRP & salivary S100A8.

3.3.7.2 Correlation between the change in salivary/systemic markers after periodontal treatment

	MMP-8 Change	HNP1-3 Change	S100A8 Change	CRP Change	IL-6 Change
MMP-8 Change		r=0.5 ***p<0.0001**	r=0.3 ***p=0.04**	r=0.1 p=0.5	r=0.1 p=0.3
HNP1-3 Change	r=0.5 ***p<0.0001**		r=0.02 p=0.9	r=0.006 p=1	r=0.3 p=0.06
S100A8 Change	r=0.3 ***p=0.04**	r=0.02 p=0.9		r= - 0.1 p=0.6	r= - 0.2 p=0.2
CRP Change	r=0.1 p=0.5	r=0.006 p=1	r= - 0.1 p=0.6		r=0.4 ***p=0.002**
IL-6 Change	r=0.1 p=0.3	r=0.3 p=0.06	r= - 0.2 p=0.2	r=0.4 ***p=0.002**	

* Statistically significant (p<0.05)

Table 3-18: Pearson correlation table demonstrates the relationship between the change of salivary and systemic markers after periodontal treatment.

As shown in Table 3-18 there were significant and moderate positive relationships in the changes (pre minus post) of salivary MMP-8 & HNP1-3 and of CRP & IL-6 after non-surgical periodontal treatment: (Pearson correlation, two-tailed p value) Figure 3-23 and Figure 3-25. In addition there was a weak, but significant relationship between the changes in salivary MMP-8 & S100A8 (Pearson correlation, two-tailed p value) Figure 3-24.

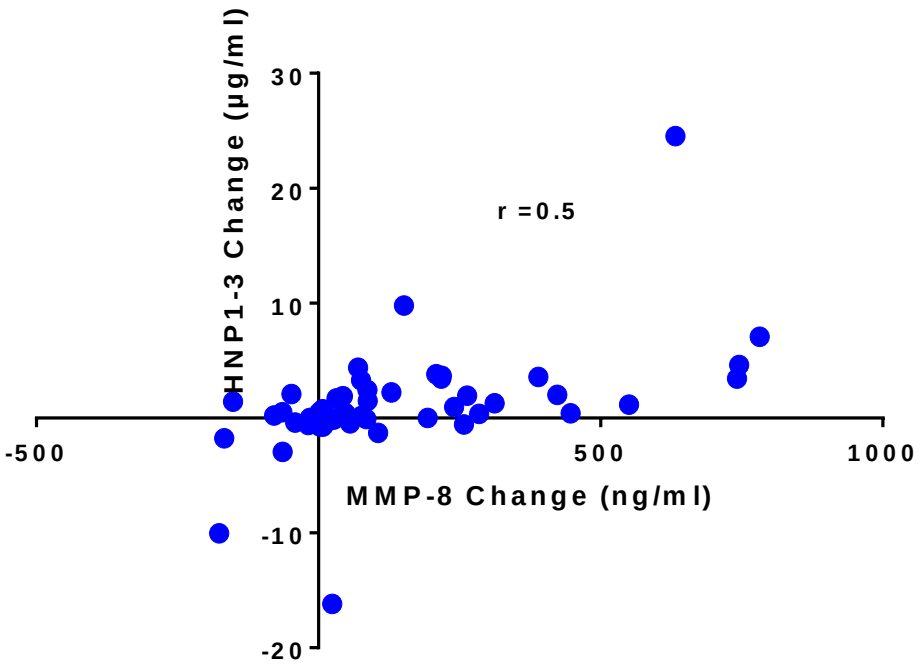

Figure 3-23: Scatter plot graph showing the significant correlation (p<0.0001) between the changes in salivary MMP-8 & HNP1-3.

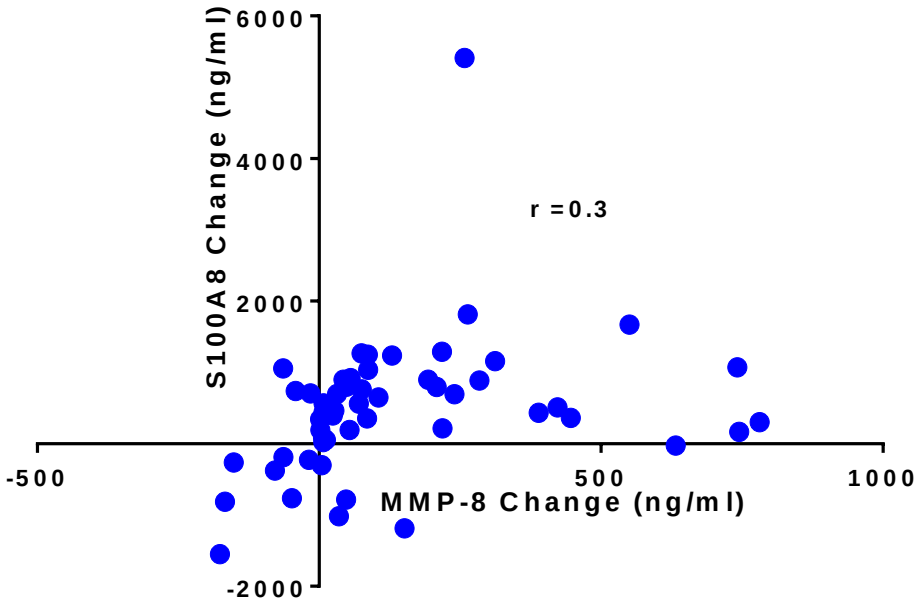

Figure 3-24: Scatter plot graph showing the significant correlation (p=0.04) between the changes in salivary MMP-8 & S100A8.

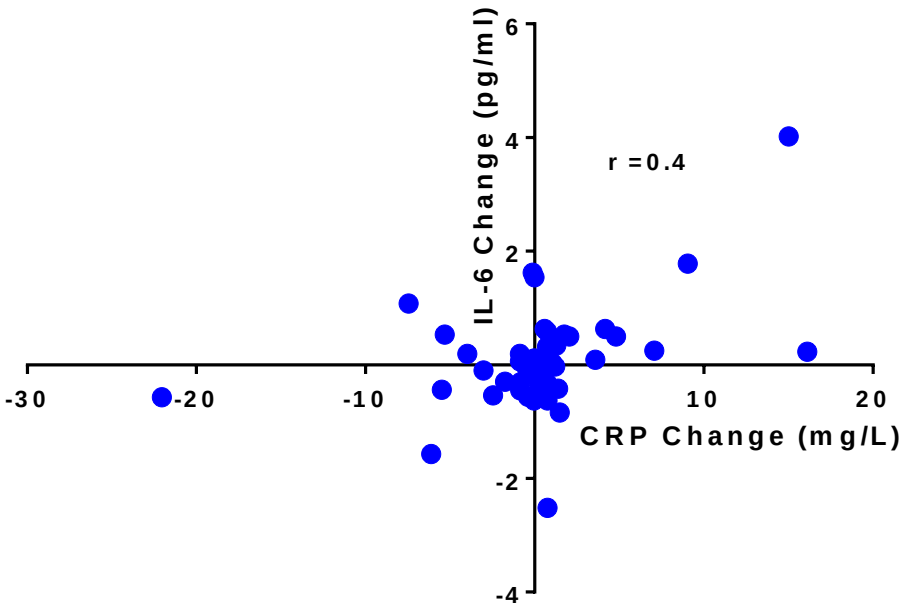

Figure 3-25: Scatter plot graph showing the significant correlation (p=0.002) between the changes in systemic CRP & IL-6.

3.3.8 Application of Salivary Biomarkers' pre-determined cut-off points into the cohort of this study

(Validation of Sensitivity Diagnostic)

In the previous chapter in this thesis, we have shown that salivary MMP-8, HNP1-3 and S100A8, individually and as a composite biomarker, had the ability to differentiate between gingivitis and chronic periodontitis.

The data from this study here was an opportunity to re-validate the ability of these salivary biomarkers (MMP-8, HNP1-3, and S100A8) to detect periodontal disease in the cohort of this study. Due to the fact that only subjects with chronic periodontitis were recruited in this study, we only can test sensitivity and not specificity of these predetermined cut-offs. Within this cohort of subjects, the application of the predetermined cut-off points for MMP-8 (>82.73 ng/ml), HNP1-3 (>3.745 µg/ml), and S100A8 (>191.3 ng/ml) at baseline detected periodontitis with sensitivity of 85%, 64.1% and 90.6% respectively.

Interestingly, when we combine the three salivary biomarkers (after normalizing each marker to its' pre-determined cut-off point), and we applied the composite marker predetermined cut-off (MMP-8+HNP1-3+S100A8) (>2.570) the sensitivity improved dramatically to 94.3%.

3.3.9 Predictors of Treatment Response

As explained before, the participants in this study were classified into responders and non-responders, based on the percentage of non-responding deep sites. A separate simple logistic regression was used to find out whether any of the study variables (explanatory variables) including age, sex, smoking, medical health, ethnicity, follow up time (in weeks), baseline bleeding index, baseline plaque index and the baseline biomarkers levels (MMP8, HNP1-3, S100A8, CRP and IL-6) predict the response status. In order to increase the power of the analysis, only predictor variables that were significant at a liberal 10% level in the individual logistic model fitted for each potential predictor variables separately were included in the final multivariate logistic model that aimed to find out the significant predictors of treatment response Table 3-19.

Predictor Variable	Effects (Odds Ratio) (OR)	95% CI for OR		P value
		LCL	UCL	
Age	-0.02 (1.02)	0.95	1.09	0.68
Gender	(1.06)	0.30	3.71	0.93
Ethnicity	(1.43)	0.40	5.06	0.58
Medical Health	(0.49)	0.13	1.86	0.30
Smoking	(0.74)	0.17	3.20	0.68
Reviewed in weeks	-0.10 (0.90)	0.71	1.16	0.42
Baseline PPD	-0.50 (0.61)	0.16	2.36	0.47
No. deep sites	-0.01 (0.99)	0.96	1.02	0.49
Baseline BI	-0.43 (0.65)	0.02	19.87	0.80
Baseline PI	-0.007(0.99)	0.04	23.97	0.99
Baseline MMP-8	-0.006(0.99)	0.99	1.00	**0.03***
Baseline HNP1-3	-0.09 (0.92)	0.75	1.12	0.39
Baseline S100A8	-0.001 (0.99)	0.99	1.00	**0.08**
Baseline CRP	-0.13 (0.88)	0.73	1.06	0.17
Baseline IL-6	-0.79 (0.45)	0.16	1.26	0.13

PPD=Probing Pocket Depth, BI=Bleeding Index, PI=Plaque Index
*** Statistically significant (p<0.05).**

Table 3-19: Summary of simple logistic regression for individual explanatory variables.

The results showed that only MMP-8 and S100A8 were significantly predicting the response status at 10% level. Therefore, further analysis was carried out by including MMP-8 and S100A8 as predictor variables and response status as outcome variable in the final multivariate logistic model.

The final analysis showed that both salivary MMP8 and S100A8 did not significantly predict the response status. However, in the individual model MMP8 baseline value was significantly predicting the response status Table 3-20.

Predictor Variable	Effects (Odds Ratio) (OR)	95% CI for OR		P value
		LCL	UCL	
MMP-8 Baseline	-0.005 (0.99)	0.99	1.00	0.08
S100A8 Baseline	0.00 (1.00)	0.99	1.00	0.66

Table 3-20: Summary of the final multivariate logistic model.

3.4 Discussion

The main purpose of this longitudinal interventional study was to investigate the prognostic utility of group of biomarkers that have been identified as potential biomarkers for the diagnosis of periodontal disease. It would be of great interest to many dental clinicians to find a tool that helps them identify patient's response to treatment beforehand, as this might be useful for treatment planning decisions and could conceivably influence selection of whether adjunctive therapeutic treatment, such as locally or systemic delivered antibiotics, are needed or not. Therefore, this prognostic test could improve and speed the quality of care to the patient as well as avoiding unnecessary over and under treatment.

The clinical results in this longitudinal study are in harmony with the overwhelming evidence that non-surgical periodontal treatment is effective in the management of periodontal disease and most patients response reasonably well to this treatment (Badersten et al., 1984; Badersten et al., 1981; Cobb, 1996; Hill et al., 1981; Hujoel et al., 2000; Lindhe et al., 1982; Pihlstrom et al., 1983; Van der Weijden and Timmerman, 2002).

Similar to Hughes and co-workers' longitudinal study, we used the percentage of non-responding deep sites as our primary outcome measure in this study, where participants were classified accordingly to responders and non-responders after treatment. The difference that we used (≥50%) cut-off instead of (≥30%) (Hughes et al., 2006b). As this cut-off (50%) suits this study data more in which it is in line with the results of many traditional studies that divided the response of periodontal patients after treatment into three categories: the majority (about 60_80%) respond extremely well to treatment, a second group (about 12-28%) continue to lose teeth after treatment (progressive PD), and a third group (about 4-10%) of patients are considered to have an 'extreme downhill' response to treatment (Goldman et al., 1986; Hirschfeld and Wasserman, 1978; McFall Jr, 1982). In this study, there were only 24.5% of the cases that were classified as not responding to the treatment. In addition, there were statistical significant reductions in the change of both probing pocket depth and number of deep sites, between responders and non-responders.

After non-surgical periodontal treatment, the salivary biomarkers MMP-8 and S100A8 concentrations reduced dramatically, While HNP1-3 did not show any

significant reduction. However, our finding of reduction of MMP-8 levels after periodontal treatment was similar to other studies (Kinane et al., 2003; Marcaccini et al., 2009b; Sexton et al., 2011). In addition, there was a significant association between both the baseline levels and change of MMP-8 and the clinical outcome indicator (PPD change) which is in line with other studies' findings (Chen et al., 2000; Kraft-Neumarker et al., 2012), while for S100A8 our data contradicted the results of Haigh and co-workers' study which concluded that S100A8 & A9 increased after treatment of periodontal disease (Haigh et al., 2010).

As well as analysing the data by the dichotomised outcome of "responders" vs "non-responders" we also investigated the correlation of biomarkers with the continuous outcome variable of change in pocket depth. These results gave similar findings to those of the primary outcome, with both MMP-8 and S100A8 being significantly but only weakly correlated with change in pocket depth.

Interestingly, MMP-8 is the only biomarker that showed a statistical difference between responders and non-responders, in both baseline levels preoperatively and changes in its concentrations postoperatively. This finding is in agreeing with Sexton and co-workers' six months case-control study in which they found MMP-8 is the best biomarker for demonstrating response to non-surgical periodontal therapy (Sexton et al., 2011). Surprisingly, the mean baseline and change in concentrations of MMP-8 were significantly higher in responders than non-responders, which indicate that the higher MMP-8 levels at baseline the better expected response to treatment. However, low levels MMP-8 (< 182.8 ng/ml) do not necessarily indicate poor response.

In terms of predicting treatment outcome of periodontal disease, none of the clinical parameters showed any predictive ability of treatment outcome which is in line with the results of studies (Hughes et al., 2006b; Persson et al., 2003). Biochemically, only MMP-8 showed prognostic ability and was able to predict the responsiveness to periodontal treatment with moderate sensitivity and specificity. Although this finding requires further validation, it gives some hope that such a periodontal biomarker with both potential diagnostic and prognostic abilities could be of help in dental settings to prioritise patients for treatment, tailor a treatment plan for every patient and also help in epidemiological screening for periodontal disease. This finding supports the suggestion that MMP-8 may be useful as a prognostic marker of periodontal disease (Passoja et al., 2008; Ramseier et al., 2009).

In this study cohort, we did not exclude smokers and diabetics and we had 3 diabetic and 14 smokers, out of 53 participants that were included in the study. Interestingly, all the diabetic and 11 smokers did respond well to the treatment and were assigned as responders. We previously showed that the higher MMP-8 baseline levels the better the response to treatment and because the majority of smokers were responders (11 out of 14) therefore, this finding applies to smoking responders as well. This contradicts the findings of Leppilahti and co-workers that smokers with elevated levels of MMP-8 at baseline predict a poor treatment response (Leppilahti et al., 2014). Despite the small number of smokers, their generally good response to periodontal treatment was not expected due to the fact the smoking is a major risk factor for periodontal disease, and the negative effect of smoking on all aspects of periodontal treatment is confirmed and established in the literature (Anner et al., 2010; D'Aiuto et al., 2005c; Grossi et al., 1996; Heasman et al., 2006; Hughes et al., 2006a; Kinane and Chestnutt, 2000; Palmer et al., 1999; Preber and Bergström, 1986; Tonetti et al., 1995).

In addition, there was no significant difference between smokers and non-smokers in terms of all the clinical and biochemical outcomes apart from the change in PI in which smokers showed significantly less PI change than non-smokers. The logical explanation for these unexpected clinical/ biochemical outcomes for smokers is the small sample size (only 14 out of 53), the large number of factors (variables) included and the marked biological variability between individuals.

The biochemical findings were also potentially in contrast to many studies such as that of Ohsawa and co-workers which reported that CRP levels were higher in smokers than non-smokers and tend to decrease following long-term smoking cessation (≥ 5 years) (Ohsawa et al., 2005). Furthermore, a previous study has found that MMP-8 levels were higher in smokers than non-smokers, due to the fact that smoking modulates the MMP-8 expression in periodontal tissues (Liu et al., 2006). On the other hand, many other studies have reported that tobacco smoking has been related to lower oral fluid MMP-8 concentrations compared with non-smokers with chronic periodontitis (Ding et al., 1994; Heikkinen et al., 2010; Mäntylä et al., 2006; Özçaka et al., 2011).

Non-surgical periodontal therapy has been used in many studies as a reliable model to study systemic inflammation (D'Aiuto et al., 2005b, 2007; D'Aiuto et al., 2004). Many studies have shown that intensive (with adjunctive local antibiotics) and successful periodontal therapy results in greater reductions in both local and

systemic inflammation than standard periodontal therapy (Artese et al., 2015; D'Aiuto et al., 2005a; D'Aiuto et al., 2006; D'Aiuto et al., 2004), particularly a significant reduction of the inflammatory systemic markers CRP & IL-6, and therefore may reduce CRP- associated cardiovascular risk (D'Aiuto et al., 2004; D'Aiuto et al., 2004; Marcaccini et al., 2009a; Mattila et al., 2002), and CRP level is directly associated with the periodontal status of an individual (Pederson et al., 1995). However, our study results showed no significant change of serum IL-6 after periodontal treatment, which was in agreement with D'Auito & co-workers' results that IL-6 only reduced in the group that received intensive and not standard treatment (D'Aiuto et al., 2005a), and another Japanese study (Yamazaki et al., 2005).

Another intervention study by Marcaccini and co-workers included cohorts of 20 controls and 25 chronic periodontitis subjects in order to investigate the difference in systemic IL-6 & CRP levels between these cohorts, as well as investigating the change in serum levels of IL-6 and CRP three months after non-surgical periodontal treatment. Interestingly they concluded that only IL-6 levels were significantly higher in diseased than control group, while both systemic markers reduced significantly after treatment (Marcaccini et al., 2009a). Similar findings were shown in a later interventional study that included 78 subjects with chronic periodontitis and 40 healthy controls and they found that both CRP & IL-6 were significantly higher in diseased group than controls. Also, the periodontal treatment provided involved non-surgical, surgical as well as antibiotics were prescribed after the surgery. Therefore, the result of the significant reduction of CRP & IL-6 three months after treatment might be largely due to administration of antibiotics (Nakajima et al., 2010).

On the other hand, others, including this study, showed that there was no significant change of serum markers after non-surgical periodontal treatment of periodontitis (Michalowicz et al., 2009; Tüter et al., 2007), as well as that these markers are not good predictors for periodontal disease progression. In addition, a systematic review and meta-analysis by Ioannidou and co-workers of the effect of periodontal treatment on serum CRP levels concluded that it is very unlikely that CRP levels can be reduced by a single round of non-surgical periodontal treatment especially in severe periodontal inflammation (Ioannidou et al., 2006).

These contradicting results, regarding the change of serum markers after periodontal treatment, of many interventional studied might be attributed to many factors such as:

1. The modalities of periodontal treatment used (Standard, Intensive with antibiotics, surgical)
2. The time elapsed since the treatment to measure these markers (Immediate, 1 month, 2months…etc),
3. The techniques used to measure the markers concentration (ELISA, Nephelometry, radial immunodiffusion assay, immunoturbidimetrics).
4. Whether the potential confounding factors were controlled or not (Smoking, age, gender, race...etc).

However, in the recent consensus report EFP/AAP workshop of periodontitis and systemic disease they concluded that the evidence showing periodontal treatment reduces CRP levels is not strong enough, and future well-designed intervention trials are still needed (Tonetti and Dyke, 2013).

Regarding the relationship between both salivary and systemic markers preoperatively, there was a significant positive moderate relationship between salivary MMP-8 & HNP1-3 as well as between serum CRP & IL-6. Postoperatively, there were significant positive moderate relationships in the change of levels of salivary MMP-8 & HNP1-3 and serum CRP & IL-6, as well as a significant positive weak relationship in the change in levels of salivary MMP-8 & S100A8. Thus the local salivary biomarkers showed a weak association with each other, as did the systemic markers with each other also.

This finding of significant correlation between CRP and IL-6 concentrations was in harmony with some other studies' findings (Ide et al., 2003; Loos et al., 2000; Ridker et al., 2000), but unlike the results reported by Ide and co-workers' finding (Ide et al., 2004).

Interestingly, there were no correlations between local and systemic markers in this study, which means that the levels of salivary biomarkers are not associated with serum markers and both sets of biomarkers are independent from each other. Therefore, the salivary biomarkers tested here were not useful as surrogate markers of systemic inflammation.

3.5 Conclusions

This interventional study has concluded that none of the clinical parameters show the ability to predict treatment outcome. On the other hand, the salivary biomarkers MMP-8 & S100A8 showed some promising periodontal prognostic ability to detect a likely good response to treatment, with MMP-8 showing the best results with moderate sensitivity and specificity. The interesting finding in the results of this study was that the higher MMP-8 concentrations before treatment the better response predicted. However, this test might be useful to detect likely good responses, but not at detecting poor responses, which would be a much more useful result clinically. In addition, a single round of standard non-surgical periodontal treatment had no effect on the systemic markers levels CRP & IL-6, and there were no correlation between salivary and systemic markers.

Therefore, these results give some hope for the use of salivary analysis as a treatment predictor of periodontal disease although the ability to detect likely poor responders might clinically be of more use than being able to predict good responses. Further studies would be valuable to test other biomarkers both separately and in combinations. The absence of any clear both clinical or biochemical prognostic marker of poor responses is consistent with other previous studies and emphasises the likely complexity of factors which are responsible for determining overall outcome in any individual patient.

CHAPTER 4- FINAL DISCUSSION, FUTURE WORK AND CONCLUSION

4. Final Discussion, Future Work and Conclusion

4.1 Final Discussion

The studies described in this thesis were carried out in order to investigate the potential role of specific salivary biomarkers as diagnostic and prognostic markers of periodontal disease. In particular, following previous studies in our lab that had identified three antimicrobial peptides, HNP1-3, LL-37 and S100A8 with diagnostic periodontal potential, we tested their potential utility as biomarkers, and additionally compared their performance with salivary MMP-8 concentrations. In a newly recruited cohort of patients of specified periodontal diagnoses, we showed that MMP-8, HNP1-3 and S100A8, but not LL-37, all were able to discriminate between gingivitis and chronic periodontitis with over 90% specificity and good sensitivity. Combining the results of these individual tests, particularly by the normalised data method described in Chapter 2 gave further improvements in the diagnostic accuracy of these tests. From these results we were able to define cut-off points for a positive test and apply these to a validation study carried out on samples from an independent cohort of subjects.

The samples for the validation study on the independent cohort were from a study investigating the effect of diabetes on periodontal disease carried out at the University of Glasgow (Hodge et al., 2012). This cohort included non-diabetic, well controlled and poorly controlled type I diabetic patients. In general these subjects had relatively low levels of periodontal disease, particularly in the non-diabetic subjects, and represented a challenging cohort for the diagnostic tests.

In general our biomarker tests performed much less well in the validation study, with biomarkers continuing to show reasonable specificity but with very low sensitivity. Furthermore we found that diabetes influenced the levels of MMP-8 and possibly S100A8 which suggests that these diagnostic tests would not be suitable for use on patients with Diabetes.

Interestingly, when we applied our pre-chosen diagnostic cut-off points to the prognostic study cohort of this study at the baseline of that study (included only cases with CP), both MMP-8 and S100A8 showed high sensitivity whilst the sensitivity for HNP1-3 was moderate. In the absence of a gingivitis group in the

prognosis study we were unable to assess specificity. In addition, in a relatively small unpublished study by Dr. F.Saleh in our laboratory (N = 35) investigating the correlation between HNP1-3 levels and the number of PMNs, he applied our HNP1-3 diagnostic cut-off to his study cohort (which included cases with G & CP) and it showed that this cut-off could differentiate gingivitis from chronic periodontitis with 100% specificity and 57% sensitivity.

These later observations from secondary analyses are much more encouraging than the results from our Glasgow cohort study and do encourage us to consider further validation studies in larger, non-diabetic cohorts.

It is noteworthy that using the cut-off points derived from our initial study tends to give much better specificity than sensitivity. It is debateable whether a periodontal diagnostic test should err on the side of specificity rather than sensitivity and would rather depend on the specific application that the test might be applied to. For example, a specific test would likely be of considerable use in large epidemiological studies to allow the identification of a sub-cohort with periodontitis with high certainty, but a very insensitive test might be of limited value as a diagnostic test for (eg) self-monitoring or diagnosis in non-dental settings such as physician's clinics.

Predicting the progression of periodontal disease and assessing its likely response to treatment has been a focus of periodontal research for years. Clinical and radiographical parameters that clinicians use to assess periodontal disease have little predictive ability and largely provide information about the history of the disease. In our longitudinal prognostic study, we were able to confirm that the clinical parameters such as PI, BI, PPD...etc, are poor predictors of periodontal treatment outcome.

The levels of salivary biomarkers MMP-8 and S100A8 both reduced significantly after periodontal treatment. Reductions in salivary biomarkers following treatment might be useful for longitudinal monitoring of disease status, particularly conceivably when applied as longitudinal self-monitoring by patients of their own condition in a home care setting. However all the biomarkers tested were unable to identify subjects who subsequently responded poorly to treatment. Interestingly those subjects with high baseline MMP-8 levels showed a good response to treatment. The biological reasons for this effect are not at all clear but it is possible

to speculate that those with an existing robust inflammatory response are also most likely to respond well to treatment.

The systemic factors CRP and IL-6 did not change in response to treatment. In addition local salivary biomarkers showed no correlation with the systemic factors studied. However, interpreting the results of the studies investigating the link between PD and systemic disease should be done with caution due to study heterogeneity and different periodontal case definitions used.

There was a modest but significant correlation between salivary levels of MMP-8 and HNP1-3, in both baseline (pre-operative) and change (post-operative) levels, which may be attributed to the fact that both biomarkers are mainly produced by PMNs. However the modest correlation only suggests that these biomarkers are not a close surrogate measure of PMN numbers and their production are differentially regulated by a range of mechanisms. Similarly, the systemic factors CRP & IL-6 also showed a significant but modest correlation. The absence of close correlations between biomarkers and treatment outcomes probably underlines the complexity of the factors which may determine outcomes.

Along with the advances in molecular biology the interest toward the potential use of oral biomarkers (from GCF, saliva or gingival tissue) as tools for point-of-care (POC) in periodontal diagnostics and prognostics has soared. Saliva has been proposed as the most useful sample to use for periodontal monitoring due to the fact that it is unlike blood or GCF in terms of:

1. Easy, rapid, safe and non-invasive, cost-efficient way of collection for disease screening without the need for highly trained professionals.
2. Simple processing and analysis required.
3. Salivary analytes are very stable which make it feasible to be collected by patients at home and posted to the laboratory.

It has also been reported that most of salivary analytes do not have marked clinically important cyclical rhythm (Fraser, 2001), and are relatively stable if stored for many hours at room temperature as well as throughout repeated freezing-thaw cycles (Unpublished study by J.Tebbutt).

The vast majority of longitudinal studies in periodontal biomarker research have focussed on monitoring the change of serum markers or GCF biomarkers over period of time. Longitudinal changes in salivary biomarkers have been reported

far less frequently. However investigating salivary analytes in relation to periodontal disease may be more promising and reflective of the overall oral status of an individual rather than the site-specific view the GCF supplies. In addition, the salivary proteome may reflect the GCF proteome with higher information content for biomarker discovery of periodontal disease and that may be why the predictability of biomarker detection in saliva is more than GCF. However, salivary biomarkers are probably not a direct surrogate measurement for GCF biomarkers as salivary concentrations may vary both with local concentrations of GCF analytes, but also may be affected by the extent of local inflammation which would result in increased GCF production.

Collectively, of all the biomarkers tested for both diagnostic and prognostic uses, MMP-8 appeared to be superior to the other markers tested. This is consistent with previous studies and has led to the development of Point of Care (POC) devices for assessing MMP-8 (Kinney et al., 2011; Sorsa et al., 2014; Sorsa et al., 2010; Sorsa et al., 2011). Indeed there are two chair-side MMP-8 testing devices that have now been developed for MMP-8:

1. Dip stick test which provides the result in 5 minutes (Mäntylä et al., 2006; Mäntylä et al., 2003).
2. Dento-analyzer device which applies a sandwich-based immunoassay system, and provide result in 15 minutes (Munjal et al., 2007).

Obviously, as we are still in the process of identification and validation of the “Golden Marker(s)” with diagnostic and prognostic ability that are very reliable, further studies are required to extend our observations and improve the utility of salivary diagnostics.

4.2 Future Work

Overall the results of both our diagnostic and prognostic studies are encouraging, but do not validate the biomarkers tested sufficiently to suggest translational studies to bring these tests to the chairside. Further studies are required particularly improve the reliability of the tests and demonstrate their utility.

For the diagnostic testing a further large study of validity would be very useful given the findings of our validation study, on subjects of known diabetic status and a reasonable prevalence of a range of periodontal disease diagnoses. With this in mind, there is a large cross-sectional study about to start within the Department which aims to recruit 200 participants from the Twins UK cohort based at Kings College London. This study will assess the accuracy of self-reported periodontal disease by questionnaire and will supplement this with salivary biomarker testing. Unfortunately this study is outside the timescale of this PhD project but will allow definitive validation and explore the scope for combining self –reported periodontal disease data with additional biomarker testing within a cohort who has been extensively phenotyped for medical and behavioural factors.

To improve the reliability of the current tests it would also be possible to carry out further proteomic analysis of collected samples to try to identify additional biomarkers present in saliva by mass spectrometry. In addition it would be useful to investigate these markers in the presence of other disease modifying factors, most notably smoking, which was excluded from the current studies described here. It might will be that a multifactorial model including salivary biomarkers and other parameters may have better accuracy than the result of a salivary test alone.

The results of the intervention study described in Chapter 3 seem less encouraging than our diagnostic study for identifying biomarkers that may predict prognosis. In this interventional study we also had the chance to collect plaque samples from deep sites (PPD >4mm) before and after (progressive/non-responding sites) treatment. It would be of great interest to investigate these microbial biomarkers change over periodontal treatment, which might give an additional predictor biomarkers beside salivary/GCF ones.

In the longer term, assuming we can improve the reliability of our tests, it would be useful to collaborate within a multidisciplinary team to develop POC diagnostics which could be evaluated in field settings.

4.3 Conclusion

In conclusion, the aim of developing salivary diagnostics remains an important goal which offers a number of potential applications which have been discussed in Chapter 1. Our studies evaluated the use of 3 novel potential salivary biomarkers, the AMPs HNP1-3, S100A8 and LL-37 together with the described marker MMP-8. The results are encouraging in demonstrating the potential utility of MMP-8, HNP1-3 and S100A8 but further work is required particularly to improve the sensitivity of diagnostic applications and to explore further combining these results with additional parameters. Biomarkers that give useful prognostic information remains a particular challenge for future work.

CHAPTER 5 - REFERENCES

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CHAPTER 6 - APPENDICES

6. Appendices

6.1 Chapter 2: Verification and validation of salivary biomarkers as diagnostic periodontal biomarkers

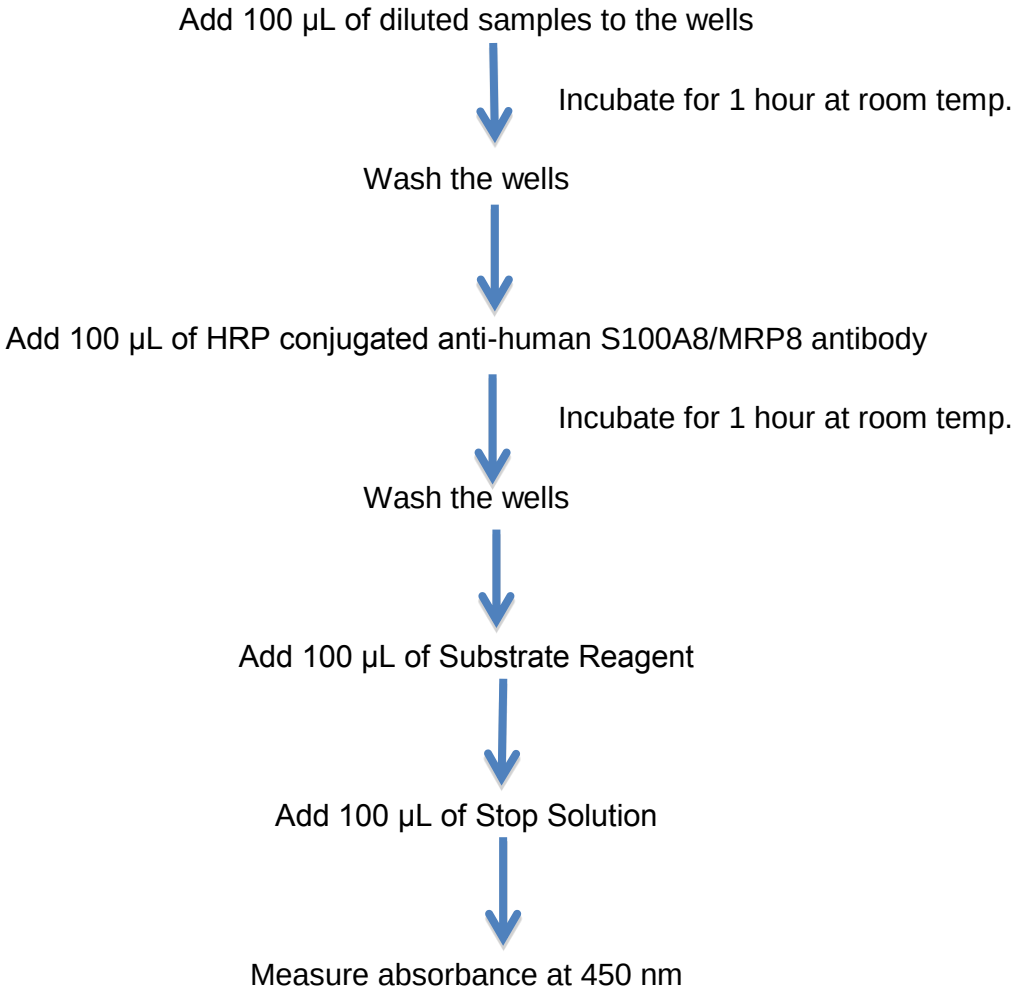
6.1.1 Salivary ELISA Protocols

6.1.1.1 HNP1-3

All the reagents were brought to room temperature (20-25 ◦ C) before use. Both the test and control samples for saliva were diluted at an optimized dilution factor of x 5000 .100µl of standard and samples were applied into appropriate wells in duplicate in a 96 well plate pre-coated with antibody. The plate was incubated for 1 hour at room temperature before the solutions were carefully emptied by inverting plate and shaking contents out then dried by tapping on a thick layer of tissues. Approximately 300µl of wash buffer was added to each well and kept for 20 seconds before emptying as above. The washing process was repeated for 3 times. Then 100µl of reconstituted biotinylated tracer antibody was added to each well using the same pipetting order as applied in previous steps and again incubated for 1 hour at room temperature. Washing was done as described above before 100µ of diluted streptavidin-peroxidase was added to each well and incubated for 1 hour at room temperature. Washing as above was repeated and 100µl of TMB substrate added to each well and incubated for 20-30 minutes at room temperature in darkness. The reaction was stopped by adding 100µl of stop solution (oxalic acid) and the plates read immediately at 450 nm using a plate reader (Optima BMG LABTECH. Germany).

6.1.1.2 S100A8

All the reagents were brought to room temperature (20-25◦C) before use. Both the test and control samples for saliva were diluted at an optimized dilution factor of x100 S100A8 (with second reading of the plates at 405nm for high concentration samples). Here is a summary of the protocol:



Optical density of the plates was read immediately at 450 nm using a plate reader (Optima BMG LABTECH.Germany).

6.1.1.3 LL-37

All the reagents were brought to room temperature (20-25 °C) before use. Both the test and control samples for saliva were diluted at an optimised dilution factor of x 5. All the steps were performed at room temperature and all samples and standards done in duplicate in an antibody pre-coated 96 well plate following the protocol represented in the schematic drawing below Figure 5-1 according to manufacturer`s instructions. Optical density of the plates was read immediately at 450 nm using a plate reader (Optima BMG LABTECH.Germany).

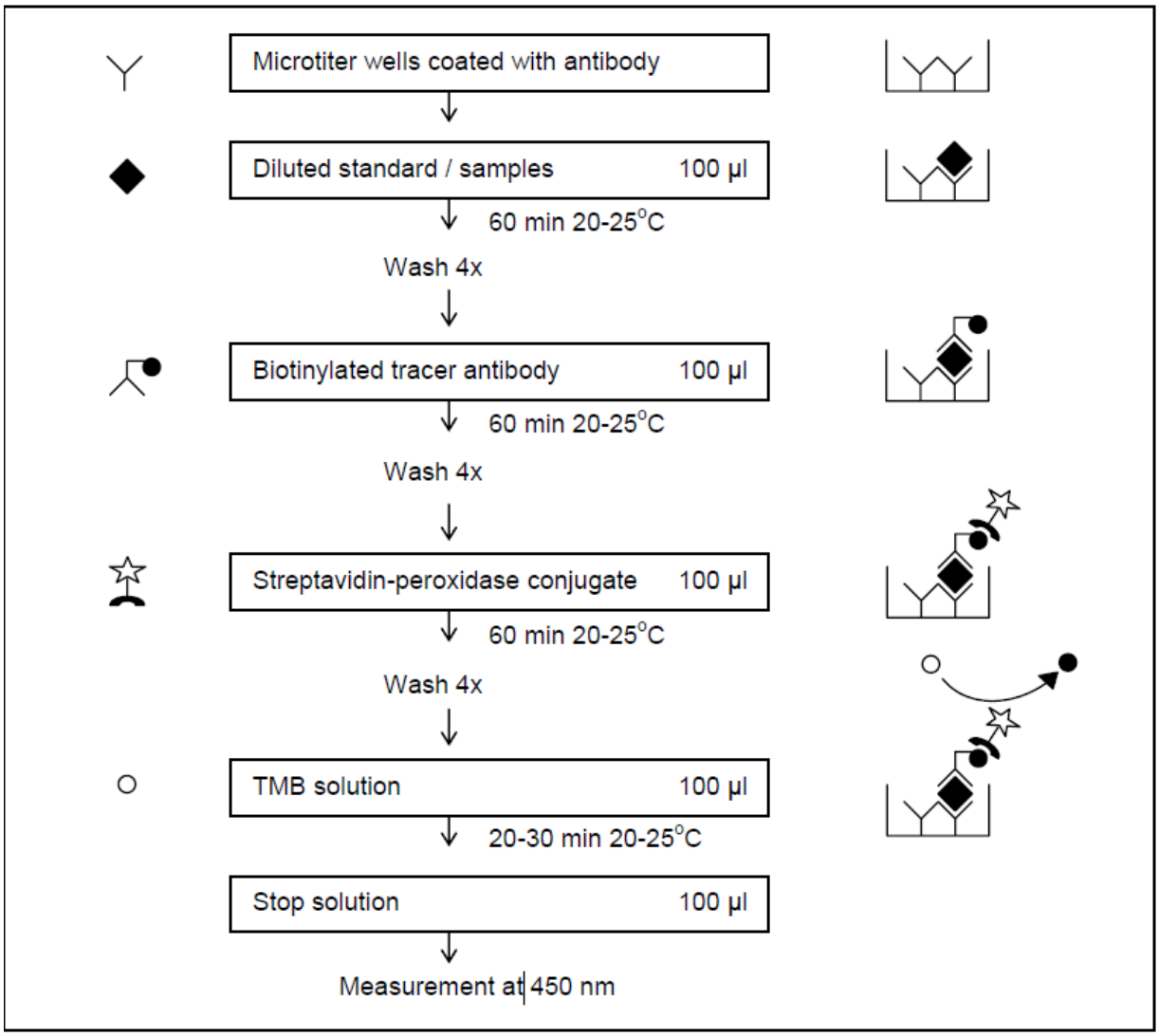


Figure 5-1: Schematic drawing of LL-37 Protocol, adopted from HK321 product information and manual of LL-37 (Hycult Biotech).

6.1.1.4 MMP-8

All the reagents were brought to room temperature (20-25 ◦ C) before use. First the plate was prepared the day before by coating the 96-well microplate with 100µl per well of the diluted Capture Antibody (Dilution Factor 1:180), the plate was sealed and incubated at room temperature overnight. Then the plate was aspirated by inverting plate and shaking contents out then dried by tapping on a thick layer of tissues and washed by 400µl of wash buffer using a squirt bottle. The wash buffer was kept for 20 seconds before emptying. The washing process was repeated for a total of 3 times. Then the plate was blocked by adding 300µl of Reagent Diluent (1mg BSA in 100ml PBS) to each well and incubated at room

temperature for a minimum of 1 hour. The plate was washed as before. Both the test and control samples for saliva were diluted at an optimized dilution factor of x 300 .100µl of standard and samples were applied into appropriate wells in duplicate in a 96 well plate pre-coated with antibody. The plate was incubated for 2 hour at room temperature, and then the plate was washed as before. 100µl of the diluted Detection Antibody (Dilution Factor 1:180) diluted in NGS (Dilution Factor 1:40) was added to each well using the same pipetting order as applied in previous steps and again incubated for 2 hour at room temperature. Washing was done as described above before. 100µ of diluted streptavidin-peroxidase (Dilution Factor 1:200) was added to each well and incubated for 20 minutes at room temperature away from direct light. Washing as above was repeated and 100µl substrate solution added to each well and incubated for 20 minutes at room temperature away from direct light. The reaction was stopped by adding 50µl of stop solution and the plates read immediately at 450 nm using a plate reader (Optima BMG LABTECH. Germany).

6.2 Chapter 3: Prognostic Biomarkers of Periodontal Disease

6.2.1 Volunteer Information Sheet

Dental Institute
at Guy's, King's College and St Thomas' Hospitals

Periodontology

Department of Periodontology
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Great Maze Pond
London Bridge
London SE1 9RT
Tel +44 (0)20 7188 4930
Fax +44 (0)20 7188 4188

KING'S College LONDON

VOLUNTEER INFORMATION SHEET

Prognostic Biomarkers of Periodontal Disease

You are being invited to take part in a study of gum health. Before you decide it is important for you to understand why the study is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

1. What is the purpose of the study?

The purpose of the study is to investigate for specific proteins within the blood and saliva that can help in the diagnosis and prediction the outcome of gum diseases. This would allow us to develop a new test to monitor patients' gum health, and may also tell us more about the disease processes going on in gum diseases

2. Why have I been invited?

You have been invited because you fit the entry criteria of being between 30 – 65 years of age and in good general health. However in order to be accepted you must:

- be free from any medical or dental conditions that may affect your own safety and well-being or that of the study staff as a result of your participation, or that may invalidate the study results;

- possess at least 20 natural teeth

- Have signs of gum disease to fit one of the different disease categories we want to test. These include signs of destructive gum disease, moderate to severe gum disease,

We aim to recruit up to 100 people to take part in this study.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. You will be given a copy of this information sheet and signed consent form to keep. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you do wish to withdraw from the study we will remove your records from the study and destroy the samples we have taken from you. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive.

4. What will happen to me if I take part?

If you decide to take part in the study you will get the chance to be treated by an experienced periodontist. Samples of saliva, blood and plaque will be collected from you before and after the gum treatment.

5. What are the possible disadvantages and risks of taking part?

There will be no anticipated risks to your health or well-being from participating in this study. All procedures and assessments will be carried out by experienced and appropriately qualified personnel. Sample of saliva is entirely painless and should take about 5 minutes in total to carry out. Taking a blood sample will involve slight discomfort from the needle and will take a further 5-10 minutes. Plaque sample collection is painless as well and will take less than 1 min. The gum treatment will be carried under local anaesthesia.

6. What are the possible benefits of taking part?

There are no direct benefits to you in taking part in the study apart from getting the initial gum treatment by an experienced periodontist.

7. How long will my gum treatment take?

Basically there will be four clinical sessions. The first one is for thorough gum examination assessment. The second and third sessions are for deep cleaning of your gums. The last session is for reassessment of your gum response to the provided treatment.

8. What will happen to the samples taken of my saliva, blood and plaque?

The samples will be stored in a secure freezer in our research laboratories, and then will be biochemically analysed in our laboratory to test for the presence and concentration of factors known to be produced during inflammation. At the end of the study any remaining samples will be stored and may be used for additional tests of biochemical present in them. The samples we take should be regarded as a gift to the Institution and you will not have any commercial rights over them in the unlikely event that they result in the development of a commercial product or test.

9. Who is organising and funding the research?

The co-sponsors of this study are Kings College London and Guys & St Thomas' Foundation NHS Trust. The study is being carried out by the research team at Kings College London Dental Institute. More specifically, the research will be carried out by Professor F Hughes and his research team.

10. What if something goes wrong?

Although we do not anticipate that there will be any complications, the sponsors have agreed that if you are harmed as a result of your participation in the study, you will be compensated, provided that, on the balance of probabilities, an injury was caused as a direct result of the intervention or procedures you received during the course of the study. These special compensation arrangements apply where an injury is caused to you that would not have occurred if you were not in the trial. These arrangements do not affect your right to pursue a claim through legal action. In the unlikely event that you do experience any unusual symptoms please report this immediately to Professor Hughes on 020 71884945 or 07714 336 296.

11. What will happen to the results of the research study?

The results of this study will be analysed and we expect to publish the results in a dental research journal. A summary of any published results will be placed on the department's web site.

12. Will my taking part in this study be kept confidential?

The information we collect from you in the study will be kept confidential and we will analyse samples anonymously.

If you consent to take part in this study any of your records, medical or non-medical, may be inspected by the sponsors of the study for the purpose of reviewing compliance with research protocols.

13. Who has reviewed the study?

This study has been reviewed and approved by Newcastle and North Tyneside 2 on 21st March 2012, ref number 12/NE/0122.

14. Contact for Further Information.

Should you have any questions or concerns regarding your participation in this study you may contact:

Professor Francis Hughes

Professor of Periodontology

Dental Institute

Kings College London

Floor 21 Tower Wing

Guys Hospital

Great Maze Pond

London SE1 9RT

Tel +44 (0)20 7188 4945

Fax +44(0) 20 7188 4188

Mobile 07714 336296

francis.hughes@kcl.ac.uk

If you agree to participate in this study you will be given a copy of this information sheet and a signed consent form to keep.

Thank you for taking the time to read this information sheet.

6.2.2 Consent Form

Dental Institute
at Guy's, King's College and St Thomas' Hospitals

Periodontology

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KING'S College LONDON

CONSENT FORM

Prognostic Biomarkers of Periodontal Disease

		Please initial box
1.	I confirm that I have read and understood the Volunteer Information Sheet version 2 dated 17th Feb 2012 for the above study and have had the opportunity to ask questions.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3.	I understand that my dental notes may be looked at by study monitor(s), the auditor(s), the Independent Ethics Committee and the regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have direct access to my records.	☐
4.	I understand that the samples that I have given may be stored at the end of this study and may be used for additional tests in the future.	☐
5.	I agree to take part in the above study.	☐

Subject's name (printed)	Subject's signature	Date

	Signature	Date

6.2.3 Clinical Study Report Form

Dental Institute
at Guy's, King's College and St Thomas' Hospitals

Periodontology

Department of Periodontology
Floor 21 Tower Wing
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Tel +44 (0)20 7188 4930
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KING'S College LONDON

Case Report Form

Study Name: Prognostic Biomarkers of Periodontal Disease

Patient`s Study ID:

Baseline Visit

Date:

Visit Duration:

Demographics:

Gender	Male □ Female □
Date of Birth	_ _/_ _/_ _ _ _ (Day/Month/Year)
Age	
Ethnicity	White □ Black □ Asian □ Caucasian □ Hispanic □ Other □ , Please specify:_____
Occupation	
Smoking Status	Smoker □ Non-Smoker □ How many per day ? __ How long? __ Never Smoke □
Alcohol Consumption	Yes □ How many Units per week? __ No □

Medical History*:

Are there any medical conditions to report? Yes □ No □ If “Yes” please describe below :
*Note: A separate detailed medical history chart is included in the patient`s notes.

Dental History:

Presenting Complaint	
History of Presenting Complaint	Onset: Location: Type of Pain: Frequency: Duration: Initiating Factors: Relieving Factors:
Past Dental History	Previous Dental Treatments: How regular do you visit your dentist/Hygienist?

Social History	Availability for Appointments: Social Status: Number of Children:
Family History	Any disease running in the family (Including Gum Disease)
Habits	Oral Hygiene procedure / Frequency: Diet: Para-functional Habits:

Sample Collection

Sample Type	Time	Duration	Wt bottle before collection	Wt bottle after collection
Saliva				
blood				

Examination

Extra-Oral Examination

Face Symmetry TMJ Lymph Nodes Smile Line: Low □ Medium □ High □

Intra-Oral Examination

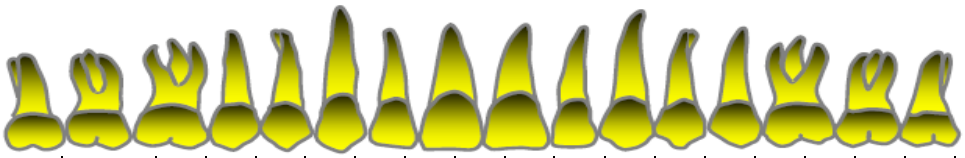
Soft Tissues Periodontal Status Gingival Biotype Plaque Index (PI)

Bleeding Index (BI)

Plaque Retentive Factors:

BPE

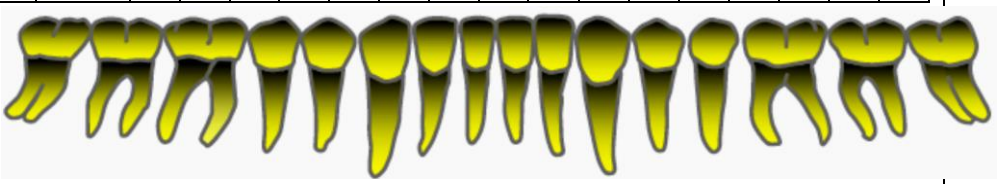
Periodontal Chart



Buccal	Date	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
Recession																	
PPD																	
Mobility																	
Furcation																	

Palatal	Date	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
Recession																	
PPD																	
Mobility																	
Furcation																	

Lingual	Date	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
Recession																	
PPD																	
Mobility																	
Furcation																	
Buccal	Date	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
Recession																	
PPD																	
Mobility																	
Furcation																	



Restorative Status

Occlusion

Special Investigations

Radiographs

DPT

PA

BW

Diagnosis

Prognosis

Initial Treatment Plane

Inclusion Criteria

Please mark the correct answer to the following questions:

	Yes	No*
1- Age		
Range 18-65 years	□	□
2-General Health		
Good general health (smokers and diabetics will be included)	□	□
3-Previous Periodontal therapy		
Should had no periodontal treatment/medication within the last 6 months	□	□
4-Consent		

Demonstrate understanding of the study and willingness to participate as evidence by voluntary written informed consent and has received a signed and dated copy of the informed consent form.	□	□
*Note: If any of the above questions are answered “No”, the subject should be withdrawn from the study as a “screen failure” on the study conclusion page.		

Exclusion Criteria

Please mark the correct answers to the following questions:

	Yes*	No
1-Complicated Medical History Any medication that can affect gingival health	□	□
2-Pergnancy Women who are known to be pregnant or who are intending to become pregnant over the duration of the study.	□	□
3-Periodontal Therapy Periodontal treatment within the last 6 months &/or antimicrobial therapy within the last 3 months.	□	□
4-Identifiable blood borne transmissible disease	□	□
*Note: If any of the above questions are answered “Yes”, the subject should be withdrawn from the study as a “screen failure” on the study conclusion page.		

Fitness and Eligibility to Participate in Study:

In the investigator`s opinion, on the basis of the screening assessment and Inclusion and Exclusion criteria at this visit, is the subject eligible and fit to participate in the study? Yes □ No □

Investigator`s signature _____ **Date** **__/__/____**

Second Visit

Date:

Visit Duration:

Subject Eligibility:

Has there been any deviation from the protocol since the last visit? Yes □ No □ If “Yes”, please record the deviation details on the comments page. Did any unwanted sign/symptoms appear or worsen since last visit? Yes □ No □ If “Yes”, please describe on the comment page. Is the subject eligible to continue in the study? Yes □ No □ If “No”, please complete the study conclusion page.

Sample Collection:

Sample Type	Time of Collection	Tooth / Site(≥5mm)
Plaque (1)		
Plaque (2)		
Plaque (3)		

Clinical Examination:

Extra Oral:

Intra Oral:

PI

BI

Work Done:

Third Visit

Date:

Visit Duration:

Subject Eligibility:

Has there been any deviation from the protocol since the last visit? Yes □ No □ If “Yes”, please record the deviation details on the comments page. Did any unwanted sign/symptoms appear or worsen since last visit? Yes □ No □ If “Yes”, please describe on the comment page. Is the subject eligible to continue in the study? Yes □ No □ If “No”, please complete the study conclusion page.

Clinical Examination:

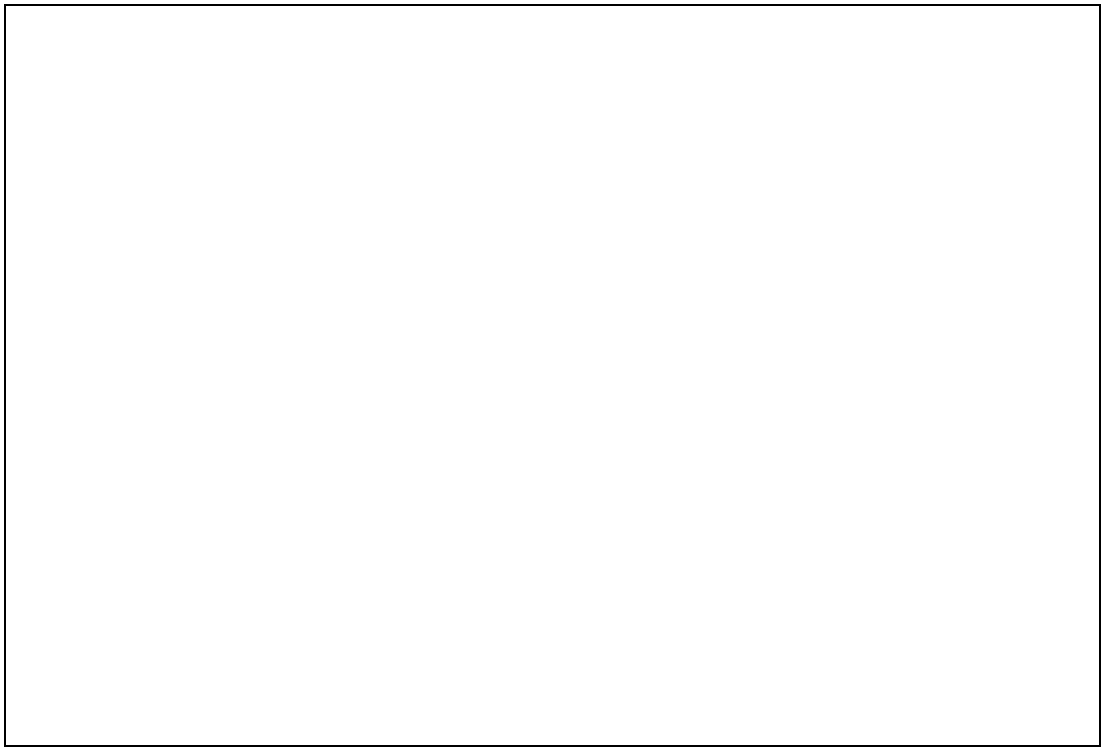
Extra Oral:

Intra Oral:

PI

BI

Work Done:



Last Visit

Date:

Visit Duration:

Subject Eligibility:

Has there been any deviation from the protocol since the last visit? Yes □ No □ If "Yes", please record the deviation details on the comments page. Did any unwanted sign/symptoms appear or worsen since last visit? Yes □ No □ If "Yes", please describe on the comment page. Is the subject eligible to continue in the study? Yes □ No □ If "No", please complete the study conclusion page.

Sample Collection

Sample Type	Time	Duration	Wt bottle before collection	Wt bottle after collection
Saliva				
blood				

Sample Type	Time of Collection	Tooth / Site(≥5mm)
Plaque (1)		
Plaque (2)		
Plaque (3)		

Clinical Examination:

Extra Oral:

Intra Oral:

PI

BI

Periodontal Chart

Use the one on the First Visit

Further Treatment Plan

Comments:

Please indicate any additional information that has not been addressed on the previous case report pages.

Page	Section Heading	Comment

Study Conclusion:

Did the subject complete the entire study? Yes □ No □

If “No” is marked, please indicate the primary reason below. Please mark only one.

Lost to follow-up □

Protocol Deviation □

Withdrawal of Consent □ Please specify:

Adverse Event □ Please specify:

Other □ Please specify:

Was there contact with the subject after the final visit? Yes □ No □

If there was a contact in relation to this study, please complete the following:

Method of Contact Telephone □ Letter □

Other □ Please specify:

Date of Last Contact: _ _/_ _/_ _ _ _ (day/month/year)

Investigator`s Signature:

I confirm that I have reviewed all the data collected in this Case Report Form and take responsibility that the information is accurate and complete.

Principal Investigator`s signature: _____ Date: __/__/_____

6.2.3 Serum ELISA Protocol

6.2.3.1 High sensitivity CRP

All the reagents were brought to room temperature (20-25 ◦ C) before use. Both the test and control samples for serum were diluted at an optimized dilution factor of x 1000 .50µl of standard and samples were applied into appropriate wells in duplicate in a 96 well plate pre-coated with antibody. The plate was incubated for 60 minutes at room temperature before the solutions were carefully emptied by inverting plate and shaking contents out then dried by tapping on a thick layer of tissues. Approximately 350µl of wash buffer was added to each well and kept for 20 seconds before emptying as above. The washing process was repeated for a total of 4 times. Then 100µl of CRP tracer was added to each well using the same pipetting order as applied in previous steps and again incubated for 60 minutes at room temperature. Washing was done as described above before 100µ of substrate solution was added to each well and incubated uncovered for 30 minutes at room temperature. The reaction was stopped by adding 100µl of Stop Solution and the plates read immediately at 405 nm using a plate reader (Optima BMG LABTECH. Germany).

6.2.3.2 High sensitivity IL-6

All the reagents were brought to room temperature (20-25 ◦ C) before use. 100µl of Assay Diluent were added to each well in the pre-coated plate. 100µl of standard and samples were applied into appropriate wells in duplicate, and incubated for 2 hours at room temperature on microplate shaker before the solutions were carefully emptied by inverting plate and shaking contents out then dried by tapping on a thick layer of tissues at least 5 times. Approximately 400µl of wash buffer was added to each well and kept for 20 seconds before emptying as above. The washing process was repeated for a total of 6 times. Then 200µl of Human IL-6 HS Conjugate was added to each well using the same pipetting order as applied in previous steps and again incubated for 2 hours at room temperature on the shaker. Washing was done as described above before 50µl of substrate solution was added to each well and incubated for 60 minutes at room temperature. Without washing the plate, 50µl of Amplifier Solution was added to each well and incubated for 30 minutes at room temperature. The reaction was

stopped by adding 50µl of Stop Solution and the plates read immediately at 490 nm using a plate reader (Optima BMG LABTECH. Germany).